University of Alberta

Tyrosyl radical-oxidized high density lipoproteins

by

Deborah Lee Merriam

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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Abstract

In striking contrast to oxidation of high density lipoprotein (HDL) using other reagents, HDL oxidized by peroxidase-generated tyrosyl radical (tyr-HDL) exhibits a markedly enhanced ability to deplete cells of cholesterol. To determine what role modification of apolipoproteins may play in this effect, cholesterol-loaded human fibroblasts were incubated with delipidated apolipoproteins in either lipid-free form or reconstituted HDL (r-HDL), and their ability to stimulate cholesterol efflux was tested. r-HDL containing the isolated apolipoproteins of tyr-HDL reproduced the ability of intact tyr-HDL to enhance cholesterol efflux when reconstituted with the whole lipid extracts of either HDL or tyr-HDL. r-HDL containing the product of oxidation of a mixture of apo A-I and apo A-II (2:1, mol:mol) by tyrosyl radical reproduced the enhancement of cholesterol efflux by tyr-HDL. Tyrosyl radical-mediated oxidation of immunoaffinity-purified HDL containing both apo A-I and apo A-II, but not immunoaffinity-purified apo A-I-only HDL or apo A-II-only HDL, produced particles with an enhanced ability to stimulate cholesterol efflux. In addition, r-HDL prepared using the apo AI-$(AII)_2$ crosslinked species stimulated cholesterol efflux to a greater extent than r-HDL prepared using other crosslinked apolipoprotein species. Taken together, these studies indicate that formation of a unique apo A-I - apo A-II heterodimer is responsible for enhanced cholesterol efflux by tyr-HDL.

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List of Symbols and Abbreviations

A	amperes
Å	angstroms
ACAT	acyl coenzyme-A:cholesterol acyltransferase
AII-only HDL	HDL containing only apo A-II
apo	apolipoprotein
BSA	bovine serum albumin
C	free (unesterified) cholesterol
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
Ci	curies
cpm	counts per minute
ctrl-apos	delipidated apolipoproteins from control HDL
Cys	cysteine
d	density
Da	daltons
DEAE-cellulose	diethylaminoethyl cellulose
DMEM	Dulbecco's modified Eagle's medium
DMEM + P/S	DMEM containing penicillin G (100 U/mL) and streptomycin (100 μg/mL)
DTPA	diethylenetriamine pentaacetic acid

EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FPLC	fast phase liquid chromatography
g	grams
h	hours
H_2O_2	hydrogen peroxide
HDL	high density lipoprotein
HPLC	high performance liquid chromatography
L	litres
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoprotein
LpAI	HDL containing only apo A-I
LpAI/AII	HDL containing apo A-I and apo A-II
M	moles/litre
MWCO	molecular weight cut-off
N	normal
NCEH	neutral cholesteryl ester hydrolase
NMWL	nominal molecular weight limit
PBS	phosphate buffered saline
PC	phosphatidylcholine
PL	phospholipids
PMSF	phenylmethylsulfonyl fluoride

r-HDL	reconstituted HDL
RCT	reverse cholesterol transport
rpm	revolutions per minute
S.D.	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SR-BI	scavenger receptor BI
TD	Tangier disease
TLC	thin layer chromatography
tyr-apos	delipidated apolipoproteins from Tyr-HDL
Tyr-HDL	tyrosyl radical-oxidized (tyrosylated) HDL
V	volts
VLDL	very low density lipoprotein
°C	degrees Celsius

(standard prefixes are: μ, micro; m, milli; k, kilo)

Chapter 1: Thesis Introduction and Literature Review

1.1 Introduction to atherosclerosis

Atherosclerosis is the principal cause of coronary artery disease and stroke, and the leading cause of death in industrialized societies (Yokota & Hansson 1995). Factors that increase a person's risk of atherosclerotic disease include hypercholesterolemia, hypertension, tobacco smoking, and diabetes mellitus (Vogel 1997). The risk of vascular disease is correlated strongly with plasma levels of apolipoprotein-B containing lipoproteins (Kannel *et al.* 1981), and even more strongly but inversely related to plasma concentrations of high density lipoprotein (HDL) cholesterol (Kannel *et al.* 1981; Miller *et al.* 1977; Stampfer *et al.* 1991).

1.1.1 The atherosclerotic lesion

In atherosclerosis, lesions containing lipid deposits form in arterial walls, decreasing their elasticity and eventually obstructing the blood vessel. Atherogenesis is generally described as occurring in three stages (Fig.1): the fatty streak lesion, the fibrous plaque lesion, and the advanced lesion (Breslow 1996; Guyton 1994; Guyton & Klemp 1996; Ross 1993).

The fatty streak lesion is characterized by the presence of lipid-filled foam cells, primarily macrophages, in the subendothelial space. These foam cells are felt to be

formed in part by unregulated uptake of oxidized and aggregated apolipoprotein B-containing lipoproteins by the macrophage class A scavenger receptor, first described in 1979 by Goldstein and colleagues (Goldstein *et al.* 1979). Both animal studies and human clinical trials suggest that fatty streak formation may be reversible (Schell & Myers 1997).

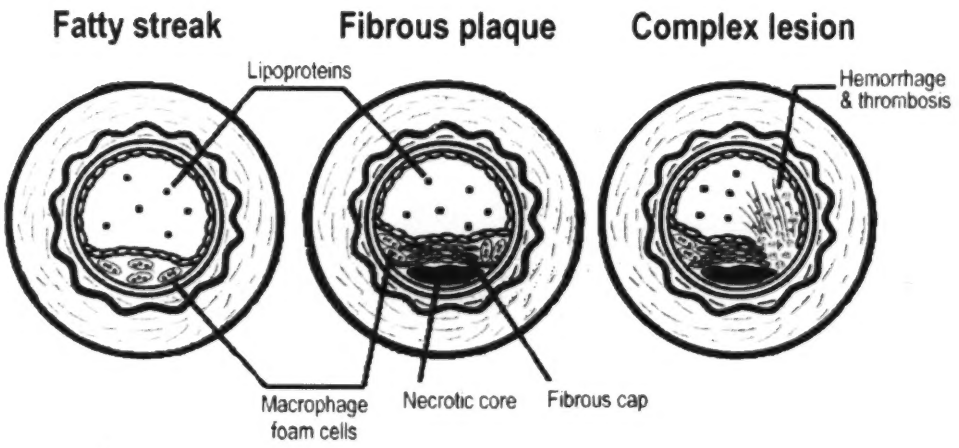


Figure 1.1. The three stages of atherosclerotic lesion formation. *Adapted from Breslow, Science (1996) 272: 685-688.*

The fibrous plaque lesion consists of a central acellular lipid core covered by a fibrous cap. The lipid core, originally thought to derive from the lysis of foam cells, is now believed to result from a combination of lipoprotein aggregation and the extrusion of lysosomes by macrophages (Guyton & Klemp 1996). The fibrous cap contains both

proliferating smooth muscle cells and collagen and elastin fibers deposited by them. Monocyte recruitment into the lesion continues at this stage.

A complex lesion is formed after the core of a fibrous plaque has undergone necrosis, calcification, hemorrhage, and/or thrombosis. Calcification, previously thought to be precipitation of calcium phosphate after cell lysis, is now believed to result from the expression of bone-related genes in macrophages and smooth muscle cells (Bostrom *et al.* 1995; Watson & Demer 1996).

Two competing hypotheses have been put forward to explain the formation of the early atherosclerotic lesion. According to the Endothelial Injury hypothesis (Ross 1986; Ross 1993), risk factors such as tobacco smoking, hypertension, and hypercholesterolemia cause endothelial injury, resulting in monocyte recruitment, lipoprotein migration into the intima, and smooth muscle cell proliferation. In contrast, the Lipid Infiltration hypothesis (Small 1988) suggests that high plasma levels of atherogenic lipoproteins lead to increased amounts of lipoprotein diffusing into the arterial intima, where they tend to accumulate and become oxidized. The oxidized lipids produced may themselves cause endothelial injury, foam cell formation, and monocyte recruitment.

1.1.2 Lipoproteins and atherosclerosis

As has already been mentioned, the risk of atherosclerosis is correlated positively with plasma levels of apolipoprotein-B containing lipoproteins (Kannel *et al.* 1981), and

inversely with plasma concentrations of HDL (Kannel *et al.* 1981; Miller *et al.* 1977; Stampfer *et al.* 1991).

Apo B-containing lipoproteins include chylomicrons, VLDL, and LDL. Many groups believe that LDL is the main atherogenic particle, based on epidemiological (Gordon *et al.* 1977; Kannel *et al.* 1981; Stampfer *et al.* 1991) and clinical trials of LDL-lowering medications (Schell & Myers 1997); it is thought that oxidation of LDL in the subendothelial or intimal space leads to its unregulated uptake by the macrophage scavenger receptor and the formation of foam cells. However, recent findings from animal models suggest that chylomicron and VLDL remnants may be the primary atherogenic particles, and recent reviews have reinterpreted the main data supporting the LDL hypothesis accordingly (Breslow 1996; Mamo *et al.* 1997). The mechanisms by which apo B-containing lipoproteins may have their atherogenic effects will be discussed in section 1.3.

Several large epidemiological studies (Gordon *et al.* 1977; Kannel *et al.* 1981; Miller *et al.* 1977) have shown that a high plasma level of HDL correlates strongly with low risk of atherosclerotic vascular disease. However, there has been a long-standing debate regarding whether a high plasma HDL concentration is directly protective against atherosclerosis. This debate may have been settled recently by a number of studies using transgenic animal models. Expression of human apo A-I in C57BL/6 mice after dietary induction of atherosclerosis increases plasma HDL levels and decreases the average lesion area (Rubin *et al.* 1991). This effect is modulated by the presence of apo A-II

(Castro *et al.* 1997; Schultz *et al.* 1993). Similarly, overexpression of human apo A-I in atherosclerosis-prone apo E-deficient mice increases plasma HDL concentrations and inhibits lesion formation (Paszty *et al.* 1994; Plump *et al.* 1994). These results correlate with studies where injection of HDL isolated from rabbit plasma into atherosclerotic rabbits induced the regression of fatty streak lesions (Badimon *et al.* 1990). Taken together, these studies suggest that HDL is directly protective against atherogenesis.

1.2 Lipoproteins as atheroprotective factors

The exact atheroprotective mechanism(s) of HDL have not been established to date, although several possible mechanisms have been proposed (Andersson 1997; Barter & Rye 1996a; Stein *et al.* 1996).

Reverse cholesterol transport (RCT) is generally accepted as the primary atheroprotective mechanism of HDL. RCT refers to the pathway for delivery of cholesterol from peripheral tissues, which are unable to metabolize cholesterol, to the liver for excretion in bile.

Other potentially atheroprotective activities of HDL include its antioxidant activity, anti-inflammatory effects, antithrombic activity, and effects on arterial tone. The abilities of HDL to protect LDL from oxidation (Decossin *et al.* 1995; Klimov *et al.* 1993; Mackness *et al.* 1991; Maier *et al.* 1994; Parthasarathy *et al.* 1990) and inhibit monocyte adhesion

to endothelial cells (Maier *et al.* 1994) may be of particular importance in preventing atherogenesis.

1.2.1 Reverse cholesterol transport

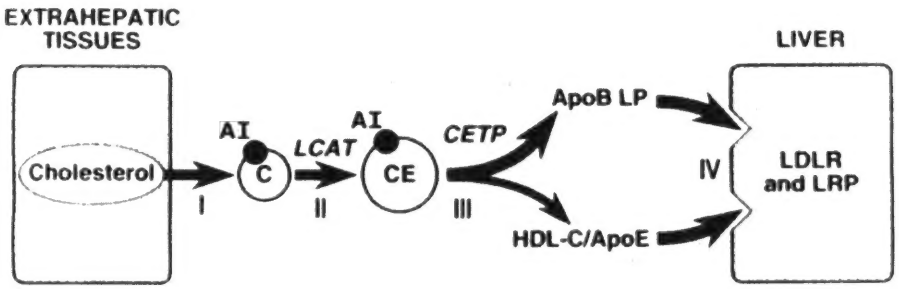


Figure 1.2. Reverse cholesterol transport. The steps involved are: I, cholesterol efflux from extrahepatic tissues; II, conversion of cholesterol (C) to cholesteryl ester (CE) by lecithin-cholesterol acyltransferase (LCAT); III, transfer of some CE to apolipoprotein B-containing lipoproteins (ApoB LP) by cholesteryl ester transfer protein (CETP); and III, uptake of C and CE by the liver. *Adapted from Dietschy, Am J Clin Nutr (1997) 65: 1581s-1589s.*

Reverse cholesterol transport refers to a pathway by which excess cholesterol is transported from extrahepatic tissues via plasma lipoproteins to the liver for excretion as bile (Barter & Rye 1996b; Dietschy 1997; Fielding & Fielding 1995a; Oram & Yokoyama 1996; Quintao 1995). This pathway, first suggested by Glomset in 1968 (Glomset 1968), represents the only means of eliminating excess cholesterol in non-steroidogenic extrahepatic tissues. RCT is usually described as having four steps.

The first step in RCT is efflux of cholesterol from cell membranes to extracellular acceptors such as HDL. Recent studies using fibroblasts from patients with Tangier Disease have demonstrated that a defect in HDL protein-mediated cellular cholesterol and phospholipid efflux is responsible for the disease's characteristics, namely low plasma concentrations of HDL and LDL, deposition of cholesteryl esters in tissues, and increased risk of atherosclerotic vascular disease (Francis *et al.* 1995; Oram & Yokoyama 1996). These findings suggest that cellular cholesterol efflux is the crucial, rate-limiting step of reverse cholesterol transport. Cellular cholesterol efflux will be described in detail in section 1.2.2.

The net effect of the second and third steps of RCT is the remodeling of HDL. In the second step, HDL cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT), causing nascent discoidal HDL particles to acquire a neutral lipid core and become spherical in shape (Fielding & Fielding 1995a). The third step consists of transfer of cholesteryl esters from HDL to apo B-containing lipoproteins by cholesteryl ester transfer protein (CETP), with a reciprocal transfer of triglycerides into HDL (Tall 1993). This transfer may cause the conversion of small spherical HDL into both larger spherical and smaller discoidal HDL particles (Tall 1993). Phospholipid transfer protein (PLTP), which transfers phospholipids from other plasma lipoproteins into HDL, has also been implicated in the remodeling of HDL (Tall 1993).

Finally, cholesterol and cholesteryl esters are delivered to the liver, where they may be repackaged into newly synthesized lipoproteins or excreted into the intestine in bile. There are three proposed mechanisms for hepatic uptake of cholesterol (Dietschy 1997): endocytosis of apo B-containing lipoproteins via the LDL receptor and the LDL receptor-related protein (LRP); endocytosis of large apo E-containing HDL by the same receptors; and selective uptake of cholesteryl esters from HDL. Robins and Fasulo have recently shown that cholesterol derived from HDL, but not from VLDL or LDL, is preferentially channeled into bile (Robins & Fasulo 1997). A putative hepatic receptor for selective uptake of cholesterol from HDL is scavenger receptor BI (SR-BI), the cDNA of which was cloned in 1994 (Acton *et al.* 1994) and identified as an HDL receptor expressed primarily in liver and steroidogenic tissues (Acton *et al.* 1996; Rigotti *et al.* 1997a). Another candidate for the hepatic HDL receptor is HB_2, which has been cloned by Fidge and colleagues (Matsumoto *et al.* 1997). In a recent study, hepatic overexpression of SR-BI in mice markedly decreased plasma HDL levels and increased biliary cholesterol concentrations (Kozarsky *et al.* 1997). Furthermore, SR-BI knockout mice have recently been shown to have markedly increased plasma HDL cholesterol content, presumably due to decreased selective cholesterol uptake (Rigotti *et al.* 1997b). These studies provide strong evidence for a role for SR-BI in RCT. However, the relative importance of SR-BI in human HDL metabolism is not yet known.

1.2.2 HDL-mediated cellular cholesterol efflux

Two principal mechanisms have been proposed for cholesterol efflux from cells. According to the first model, cholesterol diffuses from the plasma membrane to acceptor particles in extracellular fluid, down an extracellular concentration gradient maintained by LCAT (Barter & Rye 1996b; Johnson *et al.* 1991b; Rothblat *et al.* 1992). The second model suggests that a specific interaction between HDL apolipoproteins and a binding site on the cell surface induces an increase in cholesterol efflux to acceptor particles (Fielding & Fielding 1995a; Oram & Yokoyama 1996). Both of these mechanisms operate in certain experimental systems, and *in vitro* their relative contributions to cholesterol efflux appear to depend on the growth state of the cells, with aqueous diffusion accounting for any efflux from growing cells, and apolipoprotein-mediated efflux being the primary mechanism in quiescent, cholesterol-enriched cells (Mendez 1997; Oram & Yokoyama 1996).

Cholesterol can spontaneously desorb from a phospholipid membrane and diffuse through an aqueous medium to dissolve in another phospholipid membrane (Johnson *et al.* 1991b); however, this aqueous diffusion mechanism is both bi-directional and relatively slow (Fielding & Fielding 1995a). For net cholesterol efflux by this mechanism, the concentration of free cholesterol and the ratio of free cholesterol to phospholipid must be higher in the donor membrane than in the acceptor particle (Johnson *et al.* 1991b). Therefore, it has been suggested that this mechanism most likely requires phospholipid-rich HDL as acceptor particles, and that LCAT is required to maintain the concentration gradient of cholesterol from the plasma membrane to the surface of HDL (Barter & Rye 1996b; Johnson *et al.* 1991b). Ji *et al.* (1997) recently

proposed that SR-BI can tether HDL to the cell surface and promote cholesterol efflux by this mechanism, in addition to its role in mediating cholesterol ester uptake. However, even in the presence of LCAT, aqueous diffusion appears to be inefficient at clearing cellular cholesteryl ester stores (Oram & Yokoyama 1996).

The reversible binding of HDL and its apolipoproteins to high-affinity cell surface binding sites has been shown by several groups (Fidge & Nestel 1985; Mendez *et al.* 1994a; Oram *et al.* 1983), and several cell-membrane associated HDL binding proteins have been identified in extrahepatic tissues (Bond *et al.* 1991; Graham & Oram 1987; Hokland *et al.* 1992; McKnight *et al.* 1992). However, none of these binding proteins has been shown to have other properties of receptors. Rothblat *et al.* (1992) suggest that a lipid microdomain of the plasma membrane, instead of a binding protein, might be the binding site involved in stimulation of cellular cholesterol efflux.

The stimulation of cholesterol efflux from the ACAT substrate pool by HDL is dependent on the presence of intact HDL apolipoproteins (Oram *et al.* 1991). It has been proposed that lipid-free apolipoproteins bind to the cell surface after their dissociation from the HDL surface (Oram & Yokoyama 1996). Lipid-free apolipoproteins are themselves able to promote cellular cholesterol efflux, and the specificity of the interaction seems to be relatively broad: the exchangeable amphipathic α-helical apolipoproteins apo A-I, apo A-II, apo A-IV, apo E, and even insect apolipophorin III, are all able to stimulate removal of cholesterol and phospholipids from various cell types (Bielicki *et al.* 1992; Hara & Yokoyama 1991; Oram & Yokoyama 1996; vonEckardstein 1996). Synthetic

amphipathic α-helical peptides that mimic the structure of apo A-I are also able to induce cholesterol efflux (Mendez *et al.* 1994b). These observations suggest that induction of cholesterol efflux by apolipoproteins requires a particular secondary or tertiary structure rather than a primary amino acid sequence.

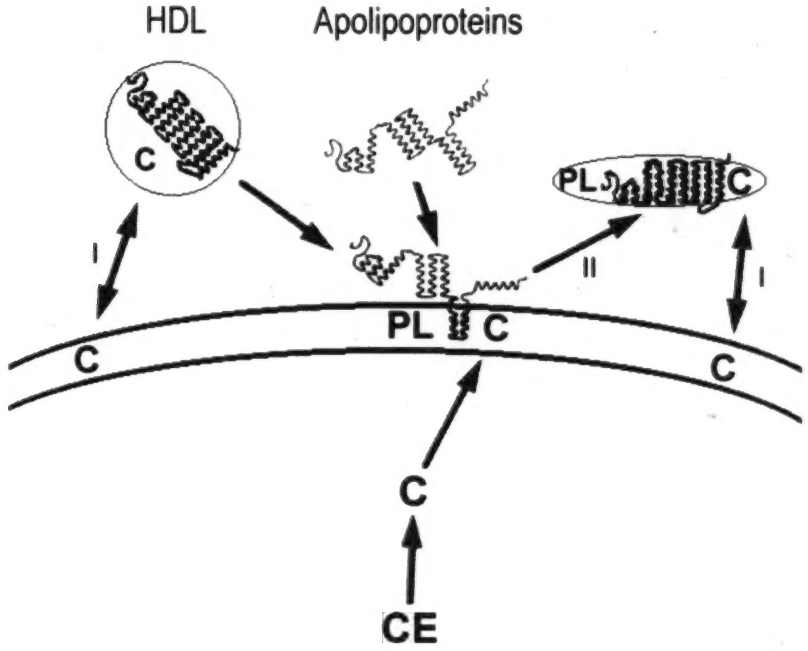


Figure 1.3. A model for two mechanisms of HDL-mediated cholesterol efflux from cells: I, diffusional (non-specific) cholesterol efflux; II, apolipoprotein-mediated (specific) cholesterol efflux. Abbreviations: C, unesterified cholesterol; CE, esterified cholesterol; PL, phospholipids. *Adapted from Oram and Yokoyama, J. Lipid Res. (1996) 37:2473-2491.*

Unlike aqueous diffusion, the apolipoprotein-mediated mechanism removes cholesterol and phospholipids from the plasma membrane in a rapid and unidirectional manner (Oram & Yokoyama 1996). Many recent studies suggest that the apolipoprotein-cell interaction also stimulates mobilization of an ACAT-accessible pool of cholesterol to specific plasma membrane domains for efflux to acceptor particles in interstitial fluid (Mendez *et al.* 1994b; Oram *et al.* 1991; Rogler *et al.* 1995; Walter *et al.* 1994a). This stimulation appears to involve a protein kinase C-mediated signal transduction pathway (Li & Yokoyama 1995; Mendez *et al.* 1991; Oram & Yokoyama 1996; Theret *et al.* 1990), and requires both an intact functional Golgi apparatus (Mendez & Uint 1996) and microtubule-dependent vesicular transport (Fielding & Fielding 1995b; Fielding & Fielding 1996). Studies by Fielding and Fielding (Fielding & Fielding 1995b; Fielding & Fielding 1996) suggest that caveolae may be the plasma membrane domains involved in this mechanism of efflux; however, this theory remains controversial (Mendez *et al.* 1997).

As previously mentioned, Tangier disease (TD) is a hereditary disorder characterized by very low plasma concentrations of HDL and apo A-I, and by deposition of cholesteryl esters in tissues (Francis *et al.* 1995; Oram & Yokoyama 1996). Fibroblast lines from unrelated TD patients showed a defect in the apolipoprotein-mediated lipid removal pathway, but the aqueous diffusion mechanism of efflux was unaffected (Francis *et al.* 1995). These results suggest that apolipoprotein-mediated lipid removal is an important means of cholesterol efflux *in vivo*, and is necessary for the generation of HDL particles.

1.2.3 Is reverse cholesterol transport atheroprotective?

Reverse cholesterol transport is generally thought to be the main mechanism for the atheroprotective effect of HDL. However, some critics have challenged this notion, based on the following evidence, suggesting instead that other properties of HDL may be responsible for its atheroprotective effects.

Apolipoprotein A-I knockout mice show no evidence of atherosclerosis despite decreased HDL levels (Li *et al.* 1993). However, the fraction of apo E associated with lipoproteins is actually increased, and the HDL of the mutants contains both apo A-IV and apo E. This result corresponds to other studies that suggest that apo A-IV and apo E can themselves form HDL and stimulate cholesterol efflux (Cohen *et al.* 1997; Hara & Yokoyama 1991; Oram & Yokoyama 1996; vonEckardstein 1996), perhaps acting as a back-up mechanism for RCT. Furthermore, another study using apo A-I knockout mice showed that the apo A-I-deficient HDL contained more triglyceride and free cholesterol, and less cholesteryl ester (Plump *et al.* 1997), and demonstrated an overall decrease in RCT that the authors attributed to decreased cholesterol efflux and LCAT activity. Taken together, these results suggest that RCT is still operating in apo A-I knockout mice at a level sufficient to protect them from atherogenesis, and therefore one cannot conclude that RCT is not the antiatherogenic mechanism of HDL from this evidence.

Barter and Rye (Barter & Rye 1996a) note that the rate of the LCAT reaction in plasma correlates negatively with the plasma HDL-cholesterol concentration, and infer that subjects with lower HDL levels would have higher rates of cholesterol esterification by LCAT - and thus higher rates of RCT. However, for this to be true, one must assume that LCAT is the rate-limiting step in RCT, as is the case in the aqueous diffusion model of cholesterol efflux. As noted above, apolipoprotein-mediated lipid removal, not aqueous diffusion, is felt to be the critical component for cholesterol efflux *in vivo*.

The role of CETP in RCT is somewhat controversial. Quintao (Quintao 1995) suggests that, since CETP transfers HDL-cholesterol into atherogenic apo B-containing lipoproteins, RCT might actually contribute to atherogenesis. Furthermore, Barter and Rye (Barter & Rye 1996a) note that CETP lowers the total HDL concentration, which suggests that RCT would be increased when HDL levels are low rather than high. On the other hand, triglyceride-rich HDL produced by CETP are susceptible to degradation by hepatic lipase, producing preβ-HDL particles which are recycled as mediators of cholesterol efflux. Transgenic mice expressing human CETP (Dinchuk *et al.* 1995) show modest increases in LDL- and VLDL-cholesterol concentrations, and decreases in plasma HDL-cholesterol concentration with an accompanying decrease in HDL particle size. Furthermore, expression of human CETP in hypertriglyceridemic apo C-III-expressing mice decreases lesion size (Hayek *et al.* 1995), although this effect appears to depend on the lipoprotein profile (Breslow 1996; Hayek *et al.* 1995). Taken together, the results from transgenic mouse studies suggest that CETP's role in promoting RCT is an atheroprotective one.

Quintao has noted that the plasma concentration of HDL appears to be independent of the human body cholesterol pool size (Blum *et al.* 1985), suggesting that HDL does not act by enhancing cholesterol clearance (Quintao 1995). Furthermore, in C57BL/6 mice expressing CETP, the flux of cholesterol from extrahepatic tissues to the liver is essentially constant regardless of the plasma HDL-cholesterol concentration, apo A-I concentration, or CETP activity (Osono *et al.* 1996). However, instead of concluding that RCT is not atheroprotective, the authors of this study suggest that the rate-limiting step of RCT resides within the extrahepatic cells themselves, at the intracellular trafficking or cholesterol efflux steps (Osono *et al.* 1996). Also, total HDL flux studies do not account for specific HDL-mediated efflux from cholesterol-laden cells in sites such as the atherosclerotic lesion.

Finally, some critics note that not all HDL deficiency syndromes are associated with atherosclerosis (Assman *et al.* 1993; Franceschini *et al.* 1980; Quintao 1995; vonEckardstein *et al.* 1995; Walter *et al.* 1994b). However, as Oram and Yokoyama suggest (Oram & Yokoyama 1996), such apparent discrepancies are easily explained by the multifactorial nature of atherogenesis: apolipoprotein-mediated RCT is atheroprotective, but other factors are required to initiate and maintain atherosclerosis.

1.3 Lipoprotein oxidation

As mentioned above, several lines of evidence suggest that oxidation of apo B-containing lipoproteins plays a major role in the initiation and progression of atherosclerosis (Berliner & Heinecke 1996; Breslow 1996; Jialal & Devaraj 1996; Mamo *et al.* 1997; Navab *et al.* 1996; Witztum & Horkko 1997).

Atherosclerotic lesions in both apo E-deficient and LDL receptor-deficient mice contain oxidized epitopes of lipoprotein particles, and these mice have high plasma levels of antibodies to oxidized lipoproteins (Palinski *et al.* 1994; Palinski *et al.* 1995). Similarly, autoantibodies to oxidized lipoproteins have been found in human atherosclerotic patients (Bergmark *et al.* 1995; Mironova *et al.* 1996; Seccia *et al.* 1997). Furthermore, antibodies against oxidized LDL stain atherosclerotic lesions from both animal models (Palinski *et al.* 1996; Palinski *et al.* 1989) and human patients (Hammer *et al.* 1995; Palinski *et al.* 1996; Yla-Herttuala *et al.* 1994). Finally, LDL eluted from both animal and human atherosclerotic lesions (Leeuwenburgh *et al.* 1997; Yla-Herttuala *et al.* 1989) contains modifications consistent with oxidation.

Oxidation of apo B-containing lipoproteins by various methods *in vitro* has been demonstrated to generate particles which bind to a number of macrophage scavenger receptors, including the type A scavenger receptor. The particles are endocytosed, generating foam cells (Goldstein *et al.* 1979; Witztum & Horkko 1997). In addition to enhanced formation of foam cells, oxidized lipids may contribute to atherogenesis by inhibiting the motility of resident macrophages, recruiting circulating macrophages by chemotaxis (Yokota & Hansson 1995), and cytotoxic effects leading to endothelial injury

(Coffey *et al.* 1995; Colles *et al.* 1996; Sevanian *et al.* 1995). Antioxidant treatment has been demonstrated to decrease lesion size in some animal models of atherosclerosis (Berliner & Heinecke 1996; Kouzuma *et al.* 1995; Tangirala *et al.* 1995) and in some clinical trials (Olsson & Yuan 1996). However, a beneficial role of antioxidant therapy in inhibiting atherosclerosis has yet to be proven (Olsson & Yuan 1996; Steinbrecher 1997).

1.3.1 Experimental models of lipoprotein oxidation

The mechanism for oxidative modification of lipoproteins *in vivo* is not well understood. However, several experimental models of lipoprotein oxidation have been proposed and are in use.

The most commonly used *in vitro* model of lipoprotein oxidation depends on the presence of either copper or iron ions. The extracellular metal ions involved in this model may be free in solution, possibly by release from their protein-bound forms in ceruloplasmin or hemin; intracellular iron has also been proposed to mediate oxidation (Berliner & Heinecke 1996). Most tissue-culture models of lipoprotein oxidation by endothelial cells, smooth muscle cells, or phagocytes require the presence of free extracellular metal ions (Berliner & Heinecke 1996). However, it is unlikely that metal-ion mediated oxidation is the physiological mechanism of lipoprotein oxidation. Measurements of metal ion concentrations in plasma and interstitial fluid (Mitchell *et al.* 1995) suggest that the concentrations normally present in vivo are much lower (10^{-18} M

in adult plasma) than those used for oxidation in vitro (1-10 μM). Furthermore, individuals with hemochromatosis or Wilson's disease are not at increased risk of atherosclerosis, despite high concentrations of iron or copper in their plasma (Berliner & Heinecke 1996).

Other *in vitro* models of lipoprotein oxidation include oxidation by reactive oxygen species such as superoxide and hydrogen peroxide (which are produced by activated phagocytes during the respiratory burst), by thiols, or by peroxynitrite (Berliner & Heinecke 1996). Thiols generated from L-cystine by smooth muscle cells are capable of autoxidation to produce superoxide. Reaction of nitric oxide with superoxide generates peroxynitrite, which promotes lipid peroxidation and nitrates protein tyrosine residues. Superoxide and other reactive oxygen species also promote lipid peroxidation in lipoproteins *in vitro*.

1.3.2 Oxidation by peroxidase-generated tyrosyl radical

A potential catalyst for lipoprotein oxidation is myeloperoxidase, an important component of the phagocyte antimicrobial and inflammatory system. In vivo, activation of phagocytes leads to a respiratory burst that produces reactive oxygen compounds, particularly superoxide ion. This activation also results in the release of myeloperoxidase from cytoplasmic granules into the extracellular space (Klebanoff 1980). Myeloperoxidase uses the hydrogen peroxide generated from superoxide to form hypochlorous acid, a potent oxidant, from chloride ion. Work done by Drs. Heinecke and

Francis has shown that myeloperoxidase also converts L-tyrosine to tyrosyl radical, another species capable of carrying out oxidative damage by the activated phagocyte (Heinecke *et al.* 1993a). Free tyrosyl radical in turn generates protein-bound tyrosyl radical by oxidizing protein tyrosine residues. The covalent interaction of free or protein-bound tyrosyl radicals leads to formation of *o,o'*-dityrosine (Figure 1.4), an intensely fluorescent product which can be used to measure tyrosyl radical formation. This reaction occurs at physiological concentrations of chloride and amino acids (Heinecke *et al.* 1993a; Heinecke *et al.* 1993b). Tyrosyl radical generated by this reaction can oxidize both proteins and lipoproteins, creating dityrosine cross-links (Heinecke *et al.* 1993a; Heinecke *et al.* 1993b), and initiating LDL lipid peroxidation (Savenkova *et al.* 1994).

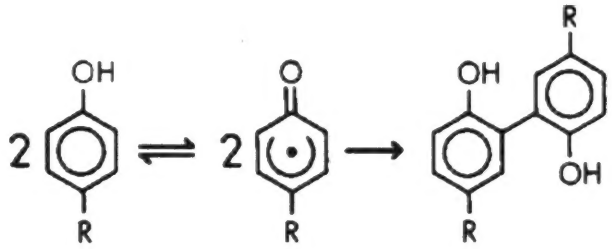


Figure 1.4. Reaction of tyrosyl radical to form *o,o'*-dityrosine. *From Francis et al., Proc Natl Acad Sci USA (1993) 90:6631-6635.*

Cultured human monocytes and neutrophils have been shown to oxidize proteins and lipoproteins using myeloperoxidase-generated tyrosyl radical (Francis *et al.* 1997; Heinecke *et al.* 1993b; Jacob *et al.* 1996), suggesting that tyrosyl radical may play a role in lipoprotein oxidation *in vivo*. Enzymatically active myeloperoxidase has been found to colocalize with monocytes and macrophages in the shoulder regions and core of fibrous

atherosclerotic plaques (Daugherty *et al.* 1994). Furthermore, levels of protein-bound dityrosine are elevated in atherosclerotic lesions (Jacob *et al.* 1996), and LDL isolated from human atherosclerotic lesions contains *o,o'*-dityrosine, but not markers of oxidation by free metal ions or hydroxyl radical (Leeuwenburgh *et al.* 1997). Taken together, these results strongly indicate a physiological role for tyrosyl radical-mediated lipoprotein oxidation in atherosclerosis.

1.3.3 Oxidation of HDL: atherogenic or atheroprotective?

There is ample evidence that HDL is susceptible to oxidation *in vivo*; in fact, HDL appears to be more susceptible to oxidation than LDL (Decossin *et al.* 1995; Klimov *et al.* 1993; Mackness *et al.* 1991; Maier *et al.* 1994; Parthasarathy *et al.* 1990). Despite this, as well as strong evidence for the atherogenic effects of oxidation of apo B-containing lipoproteins, there has been little investigation into the effects of oxidation on the function of HDL, or the presence of oxidized HDL *in vivo*.

Copper-oxidized and iron-oxidized HDL have a diminished ability to stimulate cholesterol efflux or to act as acceptors for cholesterol from cultured cells (Bonnefort-Rousselot *et al.* 1995; Morel 1994; Nagano *et al.* 1991; Rifici & Khachadurian 1996a; Rifici & Khachadurian 1996b). Similar results have been found using HDL oxidized by oxygen free radicals generated by water radiolysis (Bonnefort-Rousselot *et al.* 1995). However, as noted in section 1.3.1, these experimental models of lipoprotein oxidation are felt not to be physiologically relevant.

1.3.4 Tyrosyl radical-mediated oxidation of HDL

HDL is readily susceptible to oxidation by peroxidase-generated tyrosyl radical *in vitro* (Francis *et al.* 1993). When HDL is exposed to tyrosyl radicals, it develops apolipoprotein cross-links, other protein and lipid tyrosylation products, and low levels of lipid peroxidation products (Francis *et al.* 1993). The prominent cross-linked apolipoprotein bands seen upon SDS-PAGE have been shown by immunoblot analysis to correspond to apo A-I-(A-II)$_2$ complexes, apo A-I dimers, and apo A-I trimers (Figure 1.5) (Francis *et al.* 1993).

In striking contrast to HDL oxidized using high concentrations of copper ion or H_2O_2 or crosslinked by other means, tyrosyl radical-oxidized or "tyrosylated" HDL promotes efflux of cholesterol from cholesterol-laden cells much more effectively than does control HDL (Figure 1.6) (Francis *et al.* 1993).

Studies to determine the mechanism of this enhanced cholesterol efflux indicate that tyrosylated HDL specifically diverts intracellular cholesterol from the cholesteryl ester cycle (an "ACAT-accessible" pool) to domains on the cell surface accessible to removal by acceptor particles (an "efflux-accessible" pool) (Francis *et al.* 1996) (Figure 1.7). This effect is not caused by direct inhibition of acyl-CoA cholesterol acyltransferase (ACAT) or by stimulation of neutral cholesteryl ester hydrolase (NCEH). In addition, the abilities of control and tyrosylated HDL to act as acceptors or donors of plasma membrane

cholesterol by the diffusional pathway are identical. This suggests that tyrosylated HDL specifically stimulates a pathway for net cholesterol excretion from cells that is independent of passive cholesterol desorption.

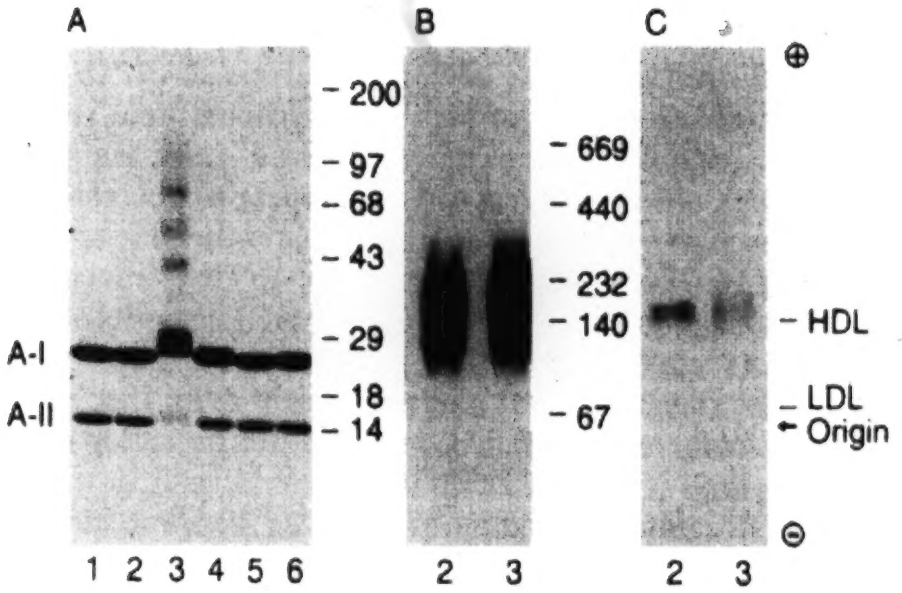


Figure 1.5. SDS/PAGE (*A*), nondenaturing gradient gel electrophoresis (*B*), and agarose gel electrophoresis (*C*) of tyrosylated HDL. Lanes: 1, HDL incubated at 4°C for 24 h; 2, HDL incubated at 37°C for 24 h; 3, HDL modified by incubation at 37°C for 24 h with complete peroxidase/H_2O_2/L-tyrosine system; 4, complete system without L-tyrosine; 5, complete system without peroxidase; 6, complete system plus catalase (5 nM). Molecular mass and lipoprotein standards are indicated. *From Francis et al., Proc. Natl. Acad. Sci. USA (1993) 90: 6631-6635.*

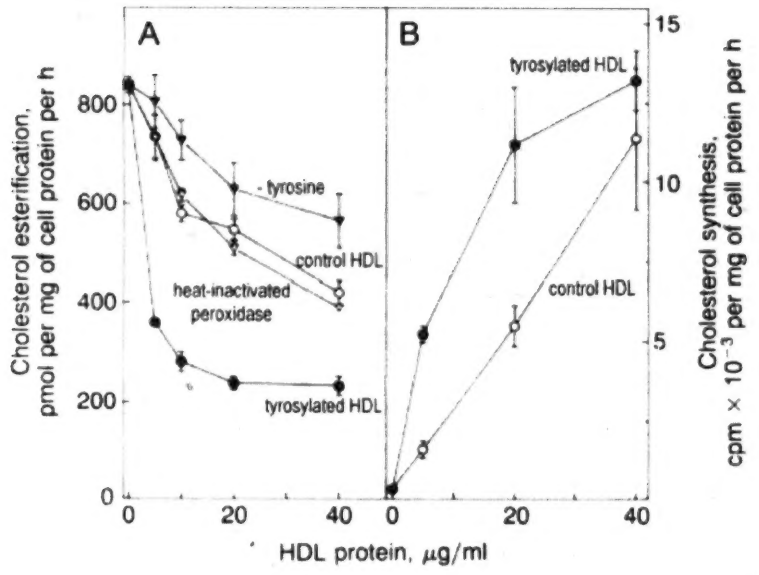


Figure 1.6. Cholesterol esterification and cholesterol synthesis in human skin fibroblasts incubated with tyrosylated HDL. (A) Confluent cultures of fibroblasts loaded with nonlipoprotein cholesterol were incubated with the indicated final concentration of HDL. After a 16h incubation at 37°C, cells were washed and incubated for 1h with medium containing [^{14}C]oleate bound to BSA at 37°C. Cellular lipids were extracted, separated by TLC, and assayed for cholesteryl [^{14}C]oleate. (B) After a 24h incubation with HDL, endogenous cholesterol synthesis was measured by exposing fibroblasts to [^{14}C]acetate in DMEM containing BSA for 2h. Cellular lipids were extracted, separated by TLC, and assayed for [^{14}C]cholesterol. *From Francis et al., Proc Natl Acad Sci USA (1993) 90:6631-6635.*

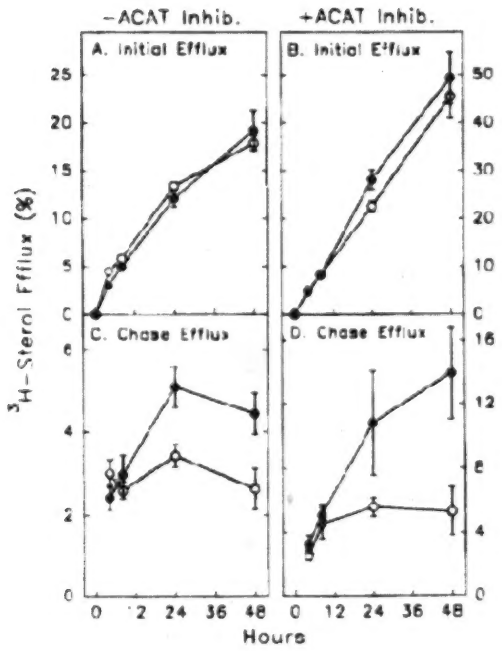


Figure 1.7. Promotion of cholesterol transport to efflux-accessible sites by HDL and tyrosylated HDL. Fibroblasts were labeled with [^{3}H]cholesterol during the last 40% of growth to confluence, then loaded with nonlipoprotein cholesterol as in Figure 1.6 in the absence or presence of 2 μg/mL ACAT inhibitor 58-035. Cells were then incubated with SFM containing 10 μg/mL HDL (open circles) or tyrosylated HDL (filled circles) for the indicated time, washed, and chased with 100 μg/mL control HDL for 1h. Media were collected after the initial incubations (A and B) and the 1h chase incubations (C and D), and aliquots were counted for radioactivity. Results are expressed as the percent of total (cell plus medium) [^{3}H]sterol in the medium for each step. *From Francis et al., Biochemistry (1996) 35:15188-15197.*

1.4 Specific Goals

The enhanced ability of HDL to promote cholesterol efflux following tyrosyl radical-mediated oxidation may be due to modification of its apolipoprotein components, its lipid components, or both. Given that the specific role of HDL in promoting cholesterol removal from cells is felt to be conferred by its apolipoproteins, I have sought to determine the role that modification of the apolipoprotein fraction of tyrosylated HDL plays in its enhanced effect on cholesterol efflux from cultured cells.

Another member of our laboratory (Dr. Wen-Qi Wang) has examined the potential role of the lipid fraction of tyrosylated HDL in this effect. His results, summarized in Appendix 1, suggest that the modified lipids do not mediate enhancement of cholesterol efflux by tyrosylated HDL.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Cell lines, growth media, antibiotics

Human skin fibroblast cells were a gift from Dr. E. L. Bierman (University of Washington, Seattle, WA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Bio-Whittaker. Fetal bovine serum was purchased from Hyclone. Trypsin-EDTA (0.5% trypsin, 5.3 mM EDTA) and penicillin/streptomycin (10000 units/mL penicillin G, 10000 μg/mL streptomycin sulfate) were purchased from Gibco BRL.

2.1.2 Chemicals and reagents

Reagents for polyacrylamide gel electrophoresis and nitrocellulose membranes for Western blotting, 10-DG desalting columns, Chelex 100 resin, and AG-11 A8 resins were purchased from Bio-Rad. High molecular weight markers, heparin sepharose resin, and Sephadex G-75 resin were purchased from Pharmacia. The ECL Western blotting detection system and Rainbow™ molecular weight markers were purchased from Amersham. Lipid assay kits (Phospholipids B, Free Cholesterol C), and egg phosphatidylcholine were purchased from Wako Pure Chemical Industries, Ltd. Albumin standard for protein assays was purchased from Pierce. DE-52 DEAE-cellulose resin and

PE SIL G polyester-backed silica plates for thin layer chromatography were purchased from Whatman. Other reagents were purchased from Sigma, Fisher, Baker, or BDH.

2.1.3 Buffers and solutions

All solutions were prepared from analytical or assured grade reagents in double distilled deionized water. Phosphate Buffered Saline (PBS), pH 7.4, contained 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8.0 g/L NaCl, and 1.144 g/L Na_2HPO_4.

2.1.4 Radioactive materials and scintillation fluors

[1-^{14}C]oleate (55-60 mCi/mmol, 100 μCi/mL) and ACS Aqueous Counting Scintillant MF were purchased from Amersham.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Media and cell culture conditions

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) containing penicillin G (100 U/mL medium), streptomycin (100 μg/mL medium), and 10% heat-inactivated fetal bovine serum (v/v) (DMEM + FBS). Fibroblasts were maintained in 75 cm^3 tissue culture flasks (Falcon) in a humidified atmosphere of 5%

CO_2, 37 °C. Once the cells reached confluence (~ 7 days), the medium was removed, and the cells were washed twice with 37 °C PBS, then incubated with trypsin-EDTA (0.05% trypsin (w/v) in PBS) for 2 minutes at 37 °C. The flask was struck with the flat of the hand to loosen cells, and the trypsinized cells were removed to a sterile 50 mL tube (Falcon) containing DMEM + FBS. The flask was washed twice with DMEM + FBS and the washes were pooled with with the trypsinized cells. Cells were centrifuged at 700 rpm for 10 minutes, then resuspended in DMEM + FBS at 10^6 cells/mL. To seed a new flask, 1 mL of resuspended trypsinized cells (10^6 cells) was added to 10 mL of DMEM + FBS in a flask, and the cells were incubated at 37 °C.

Frozen stocks were prepared by combining resuspended trypsinized cells with 10% dimethyl sulfoxide (v/v) as a cryoprotectant. Aliquots of the cells to be frozen were placed in cryovials (Wheaton). Stocks were frozen at -70 °C for 48 hours, then transferred to liquid nitrogen for storage.

Frozen stocks were thawed by incubation for 30 seconds at 37 °C, then transferred into a tissue culture flask containing 10 mL of DMEM + FBS. After incubation at 37 °C overnight, the medium was removed and replaced with fresh medium in order to remove dead cells.

2.2.2 Cholesterol esterification assay

2.2.2.1 Cell culture

Human skin fibroblasts were plated at 15000 cells/well and incubated in 1.0 mL/well DMEM+P/S containing 10% fetal bovine serum (v/v), in 24-well trays with 16 mm wells (Falcon). Fibroblasts were grown to confluence (about 7 days) in this medium in a humidified atmosphere of 5% CO_2, 37 °C.

To load the cells with cholesterol, confluent cultures were rinsed twice with 37 °C PBS-FAFA (PBS containing 1 g/L of Essentially Fatty Acid-Free Albumin, Sigma) and incubated for 48 hours in 1 mL/well of DMEM+P/S containing fatty acid-free albumin (2 mg/mL) and 30 μg/mL nonlipoprotein cholesterol (Brinton *et al.* 1986). The fibroblasts were then rinsed twice with 37 °C PBS-FAFA, and incubated overnight in 1 mL/well of DMEM+P/S containing fatty acid-free albumin (1 mg/mL) to allow equilibration of the intracellular cholesterol pools. Finally, the medium was removed and the cells were incubated for 16 - 20 hours in DMEM+P/S containing fatty acid-free albumin (1 mg/mL) and the indicated concentration of apolipoprotein, HDL particle, or reconstituted HDL (r-HDL) particle.

As a measure of cellular cholesterol remaining for esterification by ACAT, cells previously incubated with HDL or r-HDL particles were washed twice with 37 °C PBS, and then incubated for 1 hour with 0.3 mL/well DMEM containing 9 μM [^{14}C]-oleate (55-60 mCi/mmol; Amersham) bound to 3 μM BSA. At the end of 1 hour, the cells were placed on ice, the medium was removed and discarded, and the cells were rinsed twice with ice cold PBS-BSA (PBS containing 2 g/L Fraction 5 Bovine Serum Albumin;

Sigma) and twice with ice cold PBS. If extraction could not be performed immediately, the cells were stored at -20 °C for later extraction. We have previously demonstrated that a decrease in the amount of cellular cholesterol esterified with [^{14}C]-oleate by ACAT corresponds to decreased cellular cholesterol mass and to increased stimulation of cholesterol efflux (Francis *et al.* 1993; Francis *et al.* 1996).

2.2.2.2 Cellular lipid extraction and thin layer chromatography

Cellular lipids were extracted by the method of Brown *et al.* (1980). Briefly, each well was incubated for 30 minutes with 1 mL hexane/isopropanol (3/2, v/v), and the extracts were transferred into 13x100 mm borosilicate tubes (Fisher) with two 1 mL rinses. 20 µL of complete carrier (cholesteryl oleate, 0.5 mg/mL; cholesterol, 1 mg/mL; 1-monooleoyl-glycerol, 1 mg/mL; 1,2-distearoyl-glycerol, 1 mg/mL; triolein, 11 µg/mL; and oleic acid, 11 µg/mL) was added to each tube to assist with identification of individual lipid species following TLC.

Lipid extracts were dried down, resuspended in chloroform, and spotted onto the long side of 10 x 20 cm silica plates (20 x 20 cm PE SIL G plates, cut in half; Whatman) using a Romer Labs TLC AutoSpotter (Model 10; Romer Labs, Union, MO). Complete carrier (20 µL) was spotted as a standard in the outside lanes. The plates were developed in hexane/diethyl ether/acetic acid (130/40/1.5, v/v/v) to separate the neutral lipid species. Lipid bands were visualized by iodine vapour and marked for cutting. After complete evaporation of the iodine, the cholesterol ester bands were cut and placed into vials to

which 4.5 mL ACS scintilliation cocktail (Amersham) was added. [^{14}C]-Cholesteryl oleate radioactivity was quantified in a Beckman liquid scintillation counter.

2.2.2.3 Cell protein isolation and protein assay

After extraction of cellular lipids, 500 µL 0.1 N NaOH was added to each 16 mm well of cells, and the plates were agitated at room temperature for 60 minutes to solubilize the cell proteins. 50 µL aliquots were assayed for protein concentration by a microtitre version of the method of Lowry *et al.* (1951) using BSA standards of 0.3125, 0.625, 1.25, 2.50, 5.00, and 10.00 µg. Absorbance readings at 750 nm were obtained using a SpectraMAX 250 plate reader (Molecular Devices, Sunnyvale, CA), and concentrations were determined from the standard curve using SpectraMAX Pro software.

2.2.3 Lipoprotein preparation

2.2.3.1 Isolation by density gradient ultracentifugation

HDL_3 (hereafter referred to as HDL) was prepared by sequential density gradient ultracentrifugation (Chung *et al.* 1980). Briefly, pooled plasma of healthy male volunteers (4 mM EDTA) was treated with 1 µM phenylmethylsulfonyl fluoride (PMSF) to prevent lipoprotein degradation by proteases, then adjusted in density to 1.125 g/mL using KBr, loaded into polyallomer tubes (Beckman), and centrifuged at 50000 rpm for 24 hours at 8 °C in the Ti50.2 rotor (Beckman). The top layer, containing LDL and HDL_2 , was removed, and the density of the lower portion was adjusted to 1.21 g/mL using KBr and

centrifuged under the same conditions a second time. The top layer containing HDL_3 was pooled, its density was adjusted to 1.21 g/mL, and the fractions were centrifuged a third time. The top fractions were pooled, transferred to 12,000 - 14,000 MWCO dialysis membrane (#4; Spectra-Por), and dialysed against 50 mM NaCl, 5mM Tris-HCl, 1 mM EDTA, pH 7.4 at 4 °C. Finally, the HDL_3 was subjected to heparin-sepharose column chromatography (Pharmacia) to remove apo B- and apo E-containing particles (Weisgraber & Mahley 1980), dialysed once more against 150 mM NaCl, 1mM EDTA at 4 °C, and sterilized using 0.22 μm syringe filters (Millipore). Lipoproteins were stored at 4 °C under argon and used within 6 weeks of isolation.

2.2.3.2 Protease treatment of lipoproteins

Treatment of HDL or tyrosylated HDL with trypsin was performed as described by Oram *et al.* (1991). Trypsin (from bovine pancreas, 10000-13000 U/mg; Sigma) was dissolved in 0.1 M Tris, 0.01 M $CaCl_2$, pH 8.0 and added to 2 mg/mL HDL in 0.15 M NaCl, 1 mM EDTA, pH 7.2, at an enzyme-to-lipoprotein ratio of 1/40 (wt/wt). The mixture was incubated at 37 °C under argon for the specified time, then stopped by the addition of PMSF in ethanol to give a final concentration of 1 mM PMSF. The mixture was cooled to 4 °C, and the protease-treated particles were isolated from protein fragments by chromatography on a Sephadex G-75 column (1 x 25 cm; Pharmacia). To treat HDL or tyrosylated HDL with subtilisin (Subtilisin Carlsberg, from *Bacillus licheniformis*, 7-15 U/mg; Sigma), the same steps were followed, except that the reaction was stopped by the addition of protease inhibitor cocktail, to give final concentrations of 1 mM PMSF, 1mM

EDTA, 1 mM benzamidine, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 μg/mL aprotinin (North 1989). As controls, mock protease-treated particles underwent identical treatment, with 0.1 M Tris, 0.01 M $CaCl_2$, pH 8.0 being added in place of trypsin or subtilisin. Following protease treatment, HDL particle concentration was determined by phospholipid content using a modification of the method of Bartlett (Bartlett 1959; Kates 1986) with a commercial phosphorus solution (Sigma) as standard.

2.2.4 Purification of apolipoproteins A-I and A-II from HDL

2.2.4.1 Delipidation of HDL

HDL (isolated as described in section 2.2.3.1) was lyophilized, then delipidated as follows: Lyophilized lipoprotein was placed into 50 mL Teflon-capped Corex tubes, and 45 mL of diethyl ether/acetone (1/3, v/v) was added. After incubation at 4 °C overnight, the tubes were centrifuged at 3000 rpm, 4 °C for 30 minutes. The supernatant was removed and the extraction repeated twice at 4 °C for 1 hour each time. The final protein pellet was dried on low heat under nitrogen and stored at -70 °C.

2.2.4.2 Isolation of apolipoproteins A-I and A-II by column chromatography

Apolipoproteins A-I and A-II were purified as described previously by Yokoyama and coworkers (Tajima *et al.* 1983). The protein pellet was dissolved by stirring overnight at 4 °C in 25 mM Tris, 6 M urea, pH 8.0. The dissolved protein was then applied to a 30 x 1.5 cm DEAE-cellulose column (DE-52, Whatman), and eluted using a gradient of 25

mM Tris, 6 M urea, pH 8.0 to 60 mM Tris, 6 M urea, pH 8.0 formed using a gradient former (Gibco BRL). Fractions were collected using a Gilson model 203 fraction collector. Peak fractions containing apo A-I or apo A-II were identified based on absorbance at 280 nm and pooled, and the purity of the pooled apo A-I and apo A-II fractions was confirmed by SDS-PAGE. To remove all traces of urea, pooled fractions were extensively dialysed against water, then concentrated, lyophilized, and stored at -70 °C.

2.2.4.3 Dissolving delipidated apolipoproteins

Delipidated apolipoproteins were dissolved in PBS, 1 mM EDTA, pH 8.0 by incubating at 37 °C for 15 minutes, stirring at 4 °C overnight, incubating at 37 °C for an additional 15 minutes, then centrifuging in a benchtop microfuge (Eppendorf) for 5 minutes at 9000 rpm. Protein concentration of the supernatant was determined by the method of Bradford (Bradford 1976).

2.2.5 Tyrosyl radical-mediated oxidation

Buffer A was prepared by running 0.066 M KH_2PO_4, pH 8.0, over a Chelex 100 column (1.5 cm x 12.0 cm; Bio-Rad), then adding 10 mM diethylenetriamine pentaacetic acid (DTPA) for a final concentration of 100 μM. Chelex resin removes transition metal ions which might potentially catalyse lipoprotein oxidation, and DTPA inhibits metal-ion-catalysed oxidation by chelating transition metal ions.

Tyrosyl radical-mediated oxidation of HDL_3, reconstituted HDL, or delipidated apolipoproteins was performed as described by Francis *et al.* (Francis *et al.* 1993; Francis *et al.* 1996). Briefly, 1 mg protein/mL of sample was incubated in buffer A with horseradish peroxidase (100 nM; 250 units/mg; Boehringer Mannheim), L-tyrosine (100 μM), and hydrogen peroxide (100 μM) at 37 °C for 18 - 24 hours. At the end of the incubation, an aliquot was used for measurement of dityrosine fluorescence (Ex 328 nm, Em 410 nm) using a Hitachi F2000 fluorescence spectrophotometer (Amado *et al.* 1984). Dityrosine is the major product of tyrosyl radical-mediated oxidation; thus, fluorometry was used to confirm that oxidation had occurred. The remainder of the oxidized sample was subjected to size exclusion chromatography on a 10-DG column (10 mL; Bio-Rad) to remove free dityrosine and other reaction components. Protein concentration of the samples was assessed by the method of Bradford (Bradford 1976).

2.2.6 Preparation of reconstituted HDL particles

2.2.6.1 Extraction of lipid fraction from HDL or tyrosylated HDL

The whole lipid fraction of HDL or tyrosylated HDL was isolated by a modification of the method of Wang and Gustafson (Wang & Gustafson 1994). Lyophilized HDL (10 mg of HDL protein) was placed into screw-capped borosilicate tubes and incubated with 0.4 mL water and 5 mL methanol for 30 minutes; 2.5 mL chloroform was then added, and the mixture was allowed to sit for an additional 30 minutes. The samples were then centrifuged at 2000 rpm for 10 minutes. The supernatant was transferred to another tube,

and the residue was washed twice with 2 mL methanol/ chloroform (2/1, v/v). The pooled supernatants were evaporated under nitrogen. The resulting crude extract was mixed with 10 mL chloroform/methanol (2/1, v/v) and 3.75 mL 0.74% KCl (w/v), vortexed, and centrifuged at 2000 rpm for 10 minutes. The upper aqueous phase and any protein at the interface between phases was discarded, and the organic phase was evaporated under nitrogen. This step was repeated as necessary (usually 3 times) until protein precipitate no longer appeared in the organic phase during evaporation. The whole lipid extract was dissolved in 1 mL chloroform per 5 mg HDL extracted, and the phospholipid content was determined by a modification of the method of Bartlett (Bartlett 1959; Kates 1986). The lipid extract was stored under argon at -20 °C until use.

2.2.6.2 Delipidation of protein fraction from HDL or tyrosylated HDL

The delipidated whole apolipoprotein fraction from HDL or tyrosylated HDL were prepared as described in section 2.2.4.1, and dissolved as described in section 2.2.4.3.

2.2.6.3 Reconstitution by cholate dialysis

Discoidal reconstituted HDL were prepared using various delipidated apolipoproteins and egg phosphatidylcholine (PC)(Wako) and unesterified cholesterol (Sigma) using the cholate dialysis method (Jonas 1986; Matz & Jonas 1982). Briefly, aliquots of PC and cholesterol stocks (in $CHCl_3$) were placed into borosilicate tubes, and the solvent was evaporated under nitogen. The lipids were dispersed by vortexing in buffer B (10 mM

Tris-HCl, 0.15 M NaCl, 0.01% EDTA, pH 8.0). Sodium cholate (30 mg/mL in buffer B) was added to give a final PC:cholate ratio of 1:1 (mol:mol). Finally, delipidated apolipoprotein was added to give a final molar ratio of PC:cholesterol:cholate: apolipoprotein, 80:8:80:1. The lipid-cholate-apolipoprotein mixtures were stirred for 12-16 hours at 4 °C under argon, then transferred to 6000 - 8000 MWCO dialysis tubing (#1, Spectra/Por) and dialysed for 3 days against buffer B to remove cholate.

A modification of this method was used to prepare reconstituted HDL that contained the lipid extract from HDL or tyrosylated HDL (section 2.2.5.1) instead of PC and cholesterol. Aliquots of lipid extract were placed into borosilicate tubes, the solvent was evaporated, and the lipids were dispersed by vortexing in buffer B. Next, sodium cholate was added to give a final PC:cholate ratio of 1:2 (mol:mol). Finally, delipidated apolipoprotein was added to give a final molar ratio of PC:cholate:apolipoprotein, 80:160:1. The lipid-cholate-apolipoprotein mixtures were stirred and dialysed as above.

For particles which had been prepared using phosphatidylcholine and cholesterol, the phospholipid content was determined using the Phospholipids B colorimetric assay (Wako). In this assay, phospholipase D frees choline from PC, choline oxidase converts it to betaine and hydrogen peroxide, and peroxidase uses the hydrogen peroxide to convert 4-aminoantipyrene and phenol to a red quinone pigment. For particles which had been prepared using lipid extracts from HDL or tyrosylated HDL, the phospholipid content was determined by a modification of the method of Bartlett (Bartlett 1959; Kates 1986). The protein concentration was determined by the method of Bradford (Bradford 1976).

2.2.6.4 Ultracentrifugation of reconstituted particles

To remove excess free apolipoproteins, solutions of reconstituted particles were adjusted in density to 1.21 g/mL using KBr. The samples were then centrifuged at 99000 rpm for 18 hours at 4 °C in a Beckman TLA 100.3 rotor. The top 1/5 from each tube was collected, transferred to 6000 - 8000 MWCO dialysis tubing (#1, Spectra/Por), and dialysed against buffer B (10 mM Tris-HCl, 0.15 M NaCl, 0.01% EDTA, pH 8.0) to remove excess KBr.

2.2.7 Preparation of AII-only HDL by displacement of apolipoprotein A-I from HDL

Apo A-II-only HDL particles were generated by displacement of apo A-I from the surface of HDL_3 by incubation with excess apo A-II. Variants of this method have previously been used to displace exchangeable apolipoproteins from a variety of lipoproteins (Edelstein *et al.* 1982; Lagocki & Scanu 1980; Lagrost *et al.* 1995; Lagrost *et al.* 1994; Liu *et al.* 1991; Rye 1990; Vadiveloo & Fidge 1990; van Tornout *et al.* 1981). Briefly, HDL_3 in PBS + 1 mM EDTA was incubated with apo A-II at a protein ratio of HDL:apo A-II, 1:6 (wt/wt) for 2 hours at 37 °C (Lagrost *et al.* 1995). As a control, HDL_3 was also incubated in the absence of apo A-II. The density of the control and apo A-II-enriched HDL was adjusted to 1.21 g/mL using KBr, and the samples were centrifuged at 99000 rpm for 18 hours at 4 °C in a Beckman TLA 100.3 rotor to remove excess lipid-free

apolipoproteins. After ultracentrifugation, the top 1/3 to 1/2 from each tube was collected, transferred to 6000 - 8000 MWCO dialysis tubing (#1, Spectra/Por), and dialysed against buffer B (10 mM Tris-HCl, 0.15 M NaCl, 0.01% EDTA, pH 8.0) to remove the excess KBr.

2.2.8 Immunoaffinity purification of HDL subfractions

HDL subfractions were purified by immunoaffinity column chromatography with the aid of Dr. Marian Cheung (University of Washington, Seattle, WA) (Cheung & Albers 1984). We prepared HDL as described in section 2.2.3, and shipped samples in buffer C (10 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 0.02 % sodium azide, pH 7.4) at 4 °C under argon to Dr. Cheung's lab. There, the samples were incubated with anti-A-II-immunosorbent (anti-apolipoprotein A-II antibodies covalently coupled to CNBr-activated Sepharose 4B) for 1 hour at 4 °C. The immunosorbent was packed into borosilicate columns of 1.5 - 1.6 cm internal diameter, and nonbinding particles, nonspecifically bound particles, and specifically bound particles were sequentially washed from the immunosorbent using solutions of increasing acidity. The lipoproteins eluted were buffered to physiological pH with solid Tris, dialysed against buffer C, and concentrated, then shipped back to our laboratory at 4 °C under argon. Phospholipid content was determined by a modification of the method of Bartlett (Bartlett 1959; Kates 1986). Protein concentration was determined by the method of Bradford (Bradford 1976).

2.2.9 Purification of cross-linked apolipoproteins from tyrosylated HDL

2.2.9.1 Reversed phase high performance liquid chromatography

Purified apo A-I and apo A-II, tyrosyl radical-oxidized apo A-I, and delipidated HDL and tyrosylated HDL apolipoproteins were prepared as previously described, and dissolved in buffer A (section 2.2.5) as described in section 2.2.6.2. With the assistance of Dr. Paul Weers, the samples were injected onto a reversed phase HPLC column (Zorbax RX-C8, Dupont) connected to a Beckman System Gold 126NM solvent module and 166NMP detector. The proteins were then eluted with a linear AB gradient of 2% B/min, where solvent A was 0.05% (v/v) trifluoroacetic acid in water and solvent B was 0.05% (v/v) trifluoroacetic acid in acetonitrile. The gradient was controlled and absorbance at 220 nm was monitored using Beckman System Gold Nouveau software.

2.2.9.2 Fast phase liquid chromatography (FPLC)

Tyrosylated HDL apolipoproteins were delipidated and dissolved in buffer D (8 M urea, 1 mM EDTA, pH 8.0) at 23 °C as described in section 2.2.6.2. With the assistance of Dr. David Waggonner, the sample was loaded onto a MonoQ FPLC column (1 mL, Pharmacia) connected to the Pharmacia Pump P500 and Liquid Chromatography Controller LCC-500. The proteins were then eluted using a linear AB gradient of 0.5% B/min, where solvent A was buffer D, and solvent B was buffer D containing 2 M NaCl. Absorbance at 280 was monitored, and aliquots from key fractions were used for SDS polyacrylamide gel electrophoresis as described in section 2.2.10.1.

2.2.9.3 Preparative electrophoresis

Preparative gel electrophoresis was performed using a Bio-Rad Model 491 Prep Cell apparatus with 37 mm (internal diameter) gel tube assembly. For resolution of the crosslinked apolipoprotein species, a resolving gel (101 mL) of 12% acrylamide was prepared and overlaid with a stacking gel (15 mL) of 3.5% acrylamide.

Tyrosyl radical-oxidized HDL was prepared as described in section 2.2.5, then delipidated and redissolved in PBS + 1mM EDTA as described in section 2.2.6.2. Concentrated sample buffer was then added to the sample to give final concentrations of 10% glycerol (w/v), 6% SDS (w/v), 0.13 M Tris pH 6.8, and 1% Bromophenol Blue (w/v). A small aliquot was reserved for SDS-PAGE, and the remainder was loaded onto the preparative gel, and a constant voltage of 400V was applied for 26 hours at 4 °C to resolve the protein bands. The flow rate was 0.45 mL/min; 4.5 mL fractions were collected and stored at 4 °C.

To assess the separation of the crosslinked species, aliquots of every third fraction were run on SDS-PAGE (section 2.2.10.1). Based on the results of SDS-PAGE, fractions containing individual apolipoprotein species were pooled and concentrated using Ultrafree-15 centrifugal concentrators (10K NMWL; Millipore), and the purity of the pooled fractions was assessed by SDS-PAGE using silver staining.

2.2.9.4 SDS removal by AG-11 A8 column chromatography

SDS was removed from the purified crosslinked apolipoprotein species by column chromatography using AG-11 A8 ion retardation resin (Bio-Rad) (Kapp & Vinogradov 1978). Briefly, each purified sample pooled from preparative electrophoresis (section 2.2.9.3) was loaded onto a 5.0 x 1.5 cm column of AG-11 A8, then eluted with water, and 0.5 mL fractions were collected. Protein concentration was assessed by the method of Bradford (Bradford 1976), and the peak fractions were pooled and concentrated using Ultrafree-15 centrifugal concentrators (10K NMWL; Millipore).

2.2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To prepare HDL, reconstituted HDL, or apolipoproteins for SDS-PAGE, the samples were diluted 1:2 with a protein sample buffer consisting of 0.125 M Tris-HCl, pH 6.8, 20% glycerol (v/v), 4% SDS (w/v), and 0.0025% bromophenol blue (w/v). The samples were heated at 95 ℃ for 5 minutes to denature the proteins and loaded onto SDS polyacrylamide gels.

SDS-PAGE was performed using a Hoefer (20 x 20 cm) slab gel apparatus by the method of Laemmli (Laemmli 1970). For resolution of apo A-I, apo A-II, and crosslinked species, a resolving gel (30 mL) of 7 - 20% acrylamide was prepared using a gradient

former (Bio-Rad), and overlaid with a stacking gel (10 mL) of 3.5% acrylamide. A constant current of 35 mA was applied for 4 h to resolve the protein samples.

Gels were fixed in destain (40 % (v/v) methanol, 10% (v/v) acetic acid) and stained either using Coomassie Brilliant Blue R-250 (0.2% w/v in destain) or using the silver staining method of Rabilloud *et al.* (1988). Gels were photographed using the UVP gel documentation system (Diamed).

Chapter 3: Protease Treatment of Lipoproteins

3.1 Introduction

The first aim of my studies was to determine whether the enhancement of cellular cholesterol efflux by tyrosylated HDL is due to modification of HDL protein or lipid components. Previous studies had indicated that the ability of HDL to specifically deplete the pool of intracellular cholesterol available for esterification could be abolished by a brief exposure to protease (Oram *et al.* 1991). This suggested that intact HDL apolipoproteins are necessary to stimulate intracellular cholesterol efflux. Our initial experiments were designed to determine whether intact apolipoproteins in tyrosylated HDL were similarly required to promote and enhance intracellular cholesterol efflux.

Trypsin and pronase have been used for lipoprotein proteolysis in a number of studies (Borensztajn *et al.* 1982; Borensztajn & Kotlar 1984; Borensztajn *et al.* 1991; Oram *et al.* 1991; Tabas & Tall 1984). Trypsin is a mammalian serine protease which hydrolyses peptides at lysine or arginine residues. Pronase is a commercial mixture of exo- and endopeptidases isolated from a strain of *Streptomyces griseus* (Jurasek *et al.* 1976). However, pronase has no known inhibitor, and must be heated above 80 °C to be inactivated (personal communication, Sigma Technical Services). Subtilisin, on the other hand, is a bacterial serine protease with unusually broad substrate specificity (Phillip & Bender 1983), and may be inhibited using a cocktail of inhibitors (personal

communication, Sigma Technical Services). Therefore I have used trypsin as a specific protease, and subtilisin as a nonspecific protease in place of pronase.

Previous studies by Oram and colleagues (Oram *et al.* 1991) demonstrated that proteolysis using trypsin abolished the ability of HDL_3 to bind to a 110 kDa HDL binding protein, and markedly decreased the ability of HDL_3 to remove cholesterol from the pool of intracellular cholesterol available for esterification in cholesterol-loaded fibroblasts. Furthermore, prior work in our laboratory has shown that treatment of both control and tyrosylated HDL with trypsin removes their ability to compete for high-affinity HDL binding sites on the surface of cholesterol-loaded fibroblasts (Appendix 2). These binding data were recently confirmed by the results of Mendez and Oram (Mendez & Oram 1997), who demonstrated that trypsin treatment of HDL for as little as five minutes nearly abolished its interaction with high-affinity cell-surface HDL binding sites and significantly reduced its ability to remove cholesterol from ACAT-accessible cellular pools.

Based on these results, we expected that if the active component of tyrosylated HDL were contained in the apolipoprotein fraction, proteolysis would decrease its ability to promote cholesterol efflux. HDL was treated with trypsin or subtilisin either before or after oxidation by tyrosyl radical, then used in cell culture experiments.

3.2 Results

I first treated control and tyrosylated HDL with either trypsin or subtilisin for 30 minutes, as described in section 2.2.3.2, and assessed their ability to promote cholesterol efflux using the cholesterol esterification assay (section 2.2.2). Briefly, cholesterol-loaded fibroblasts were incubated with control, tyrosylated, proteolysed, and proteolysed tyrosylated HDL for 16-20 hours and then incubated with [^{14}C]-oleate for 1 hour. Formation of labeled cholesteryl ester during this 1 hour period was used to assess the size of the remaining pool of free cholesterol for esterification by ACAT.

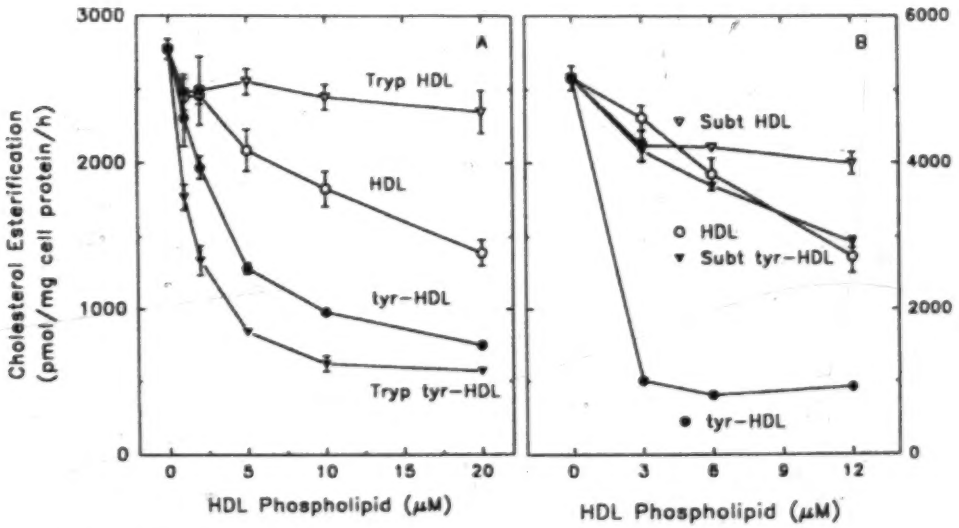


Figure 3.1. Depletion of ACAT-accessible cellular cholesterol by tyrosylated HDL treated with protease for 30 minutes. Control and tyrosylated HDL were subjected to proteolysis by trypsin (A) or subtilisin (B) for 30 minutes, and the partially digested particles reisolated. Cholesterol-loaded fibroblasts were incubated with intact or protease-treated HDL particles for 16 h, washed, and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Values are the mean +/- SD of 3 determinations and are representative of three similar experiments.

In sharp contrast to trypsin treatment of control HDL, trypsin-treated tyrosylated HDL retained its ability to deplete cells of the cholesterol pool esterified by ACAT (Fig.3.1A). Subtilisin treatment of tyrosylated HDL reduced its ability to promote intracellular cholesterol efflux, but only to the same level as intact control HDL (Figure 3.1B).

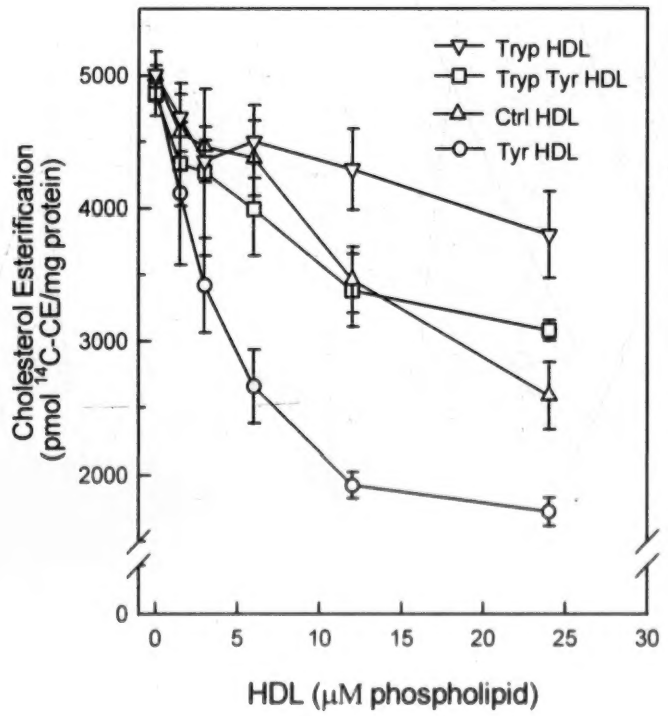
The discrepancy between the results obtained with the two proteases suggested that proteolysis of tyrosylated HDL by trypsin for 30 minutes was insufficient to decrease its ability to stimulate cholesterol efflux. Therefore, I lengthened the period of proteolysis by trypsin to 2 hours, and retested the ability of the resultant particles to promote cholesterol efflux. As shown by SDS-PAGE (Figure 3.2B), trypsin treatment of control HDL for 30 minutes left little intact apo A-I and a series of discrete protein fragments. In comparison, trypsin treatment of tyrosylated HDL for 30 minutes caused disappearance of the crosslinked apolipoprotein and apo A-I bands, and a fragment smear was seen in place of the discrete fragment bands. These results were similar to those seen for SDS-PAGE of subtilisin-treated HDL and tyrosylated HDL (Figure 3.3). Furthermore, tyrosylated HDL treated with trypsin for 2 hours showed no intact or crosslinked apolipoproteins and an even more diffuse fragment smear (Figure 3.2B). However, the 2 hour trypsin treatment of tyrosylated HDL only partially inhibited its ability to stimulate intracellular cholesterol efflux (Figure 3.2A), and trypsin digestion for up to 24 hours did not further decrease its ability to enhance efflux (Figure 3.4).

Figure 3.2 (page 50) Depletion of ACAT-accessible cellular cholesterol by tyrosylated HDL treated with protease for 120 minutes. **A**. Control or tyrosylated HDL were subjected to protease treatment with trypsin for 120 minutes and reisolated. Cholesterol-loaded fibroblasts were incubated with intact or protease-treated HDL particles for 16 h, washed, and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Values are the mean +/- SD of 3 determinations and are representative of two similar experiments.. **B**. SDS-PAGE followed by silver staining. Abbreviations: Ctrl, control HDL; Ctrl 30', control HDL treated with trypsin for 30 minutes; Tyr, tyrosylated HDL; Tyr 30', tyrosylated HDL treated with trypsin for 30 minutes; Tyr 120', tyrosylated HDL treated with trypsin for 120 minutes. Molecular mass is indicated.

Figure 3.3 (page 51) SDS-polyacrylamide gel electrophoresis of control or tyrosylated HDL treated with subtilisin, followed by silver staining. Abbreviations: LMW Stds, low molecular weight markers; Ctrl, control HDL; Subt Ctrl, control HDL treated with subtilisin for 30 minutes; Tyr, tyrosylated HDL; Subt Tyr, tyrosylated HDL treated with subtilisin for 30 minutes. Molecular mass is indicated.

Figure 3.2

A



B

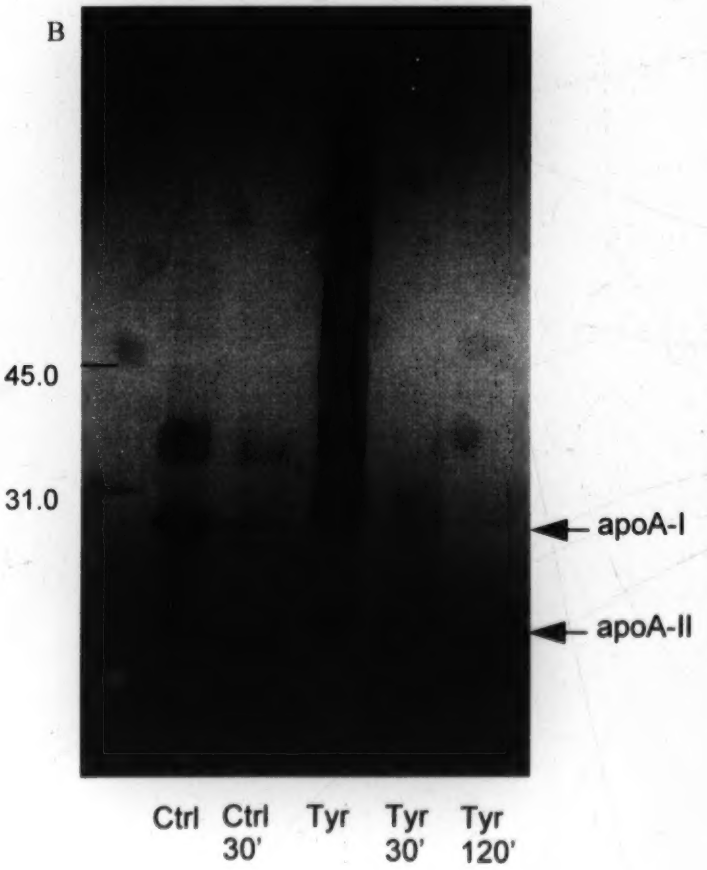


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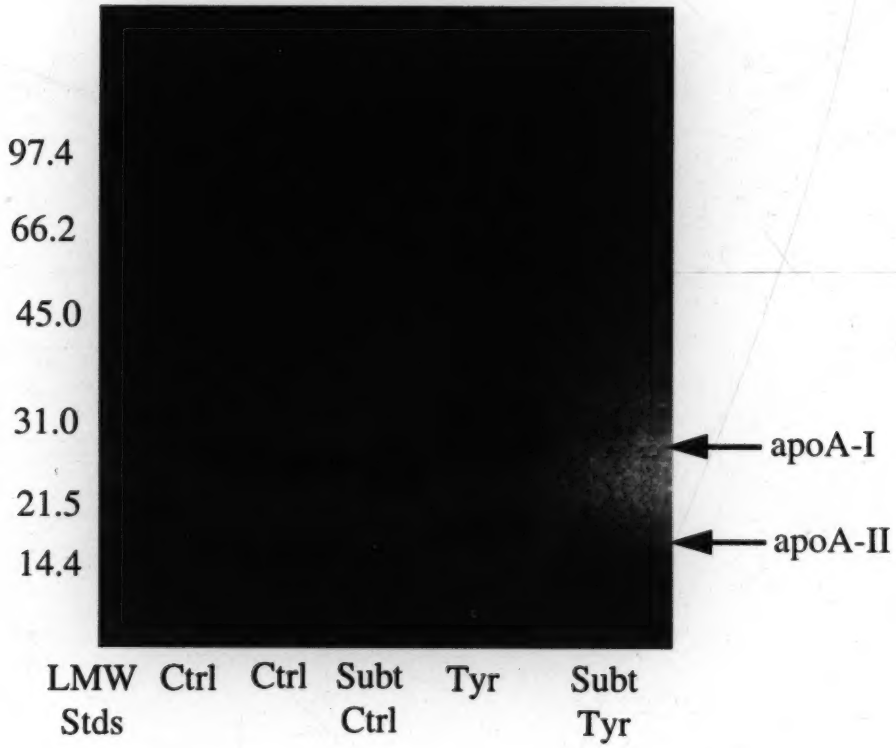
Figure 3.3: Subtilisin treatment of control and tyrosylated HDL for 30 minutes



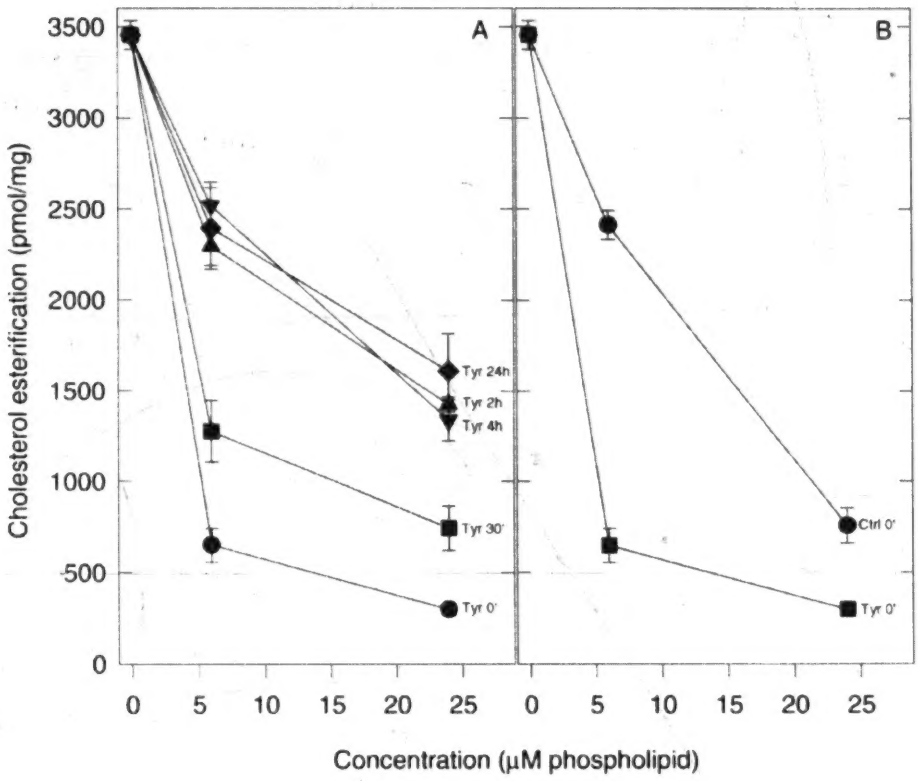


Figure 3.4. Time course of trypsin treatment of tyrosylated HDL. Tyrosylated HDL was subjected to protease treatment with trypsin for the indicated times and reisolated. Cholesterol-loaded fibroblasts were incubated with intact or protease-treated HDL particles for 16 h, washed, and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: Ctrl, control HDL; Tyr, tyrosylated HDL. Values are the mean +/- SD of 3 determinations.

To determine if intact apolipoproteins are required for tyrosyl radical-mediated oxidation to generate HDL capable of enhancing cholesterol efflux, I treated HDL with trypsin or subtilisin for 30 minutes prior to subjecting it to oxidation. Treatment of HDL with either

protease resulted in a loss of ability to stimulate cholesterol efflux. However, this was partially restored by subsequent exposure to tyrosyl radical, to half to two-thirds of the activity of tyrosylated HDL (Figure 3.5).

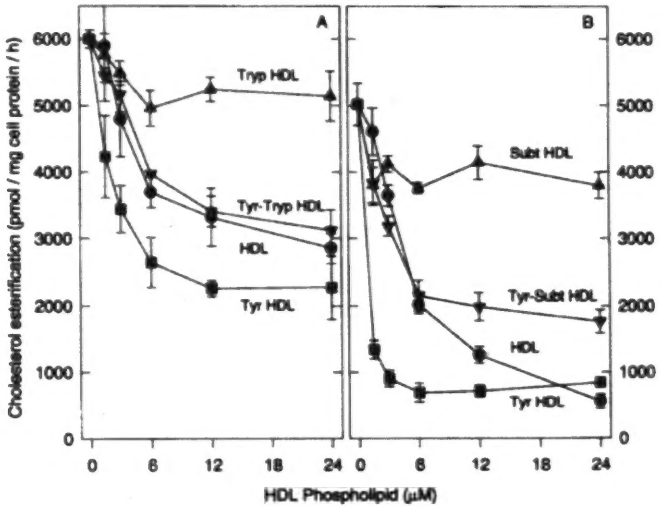


Figure 3.5. Depletion of ACAT-accessible cellular cholesterol by tyrosylated HDL previously treated with protease for 30 minutes. HDL subjected to protease treatment with trypsin (A) or subtilisin (B) for 30 minutes were reisolated and oxidized by tyrosyl radical. Cholesterol-loaded fibroblasts were incubated with control or tyrosylated HDL particles for 16 h, washed, and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Values are the mean +/- SD of 4 determinations and are representative of two similar experiments.

3.3 Summary

In summary, brief trypsin treatment of tyrosylated HDL does not alter its ability to stimulate cholesterol efflux, while treatment with subtilisin or extended treatment with

trypsin reduces, but does not abolish, its ability to stimulate cholesterol efflux. In contrast, treatment of HDL with either trypsin or subtilisin markedly decreased its ability to stimulate the removal of ACAT-accessible cholesterol.

Based on these results, we could not conclude that the apolipoprotein fraction of tyrosylated HDL is responsible for its increased ability to stimulate cholesterol efflux. The results suggest either that enhancement of cholesterol efflux by tyrosylated HDL is not due to modification of its apolipoproteins, or that some intact apolipoproteins or active apolipoprotein fragments are still present after protease treatment. Tyrosyl radical-mediated oxidation of HDL could render it less susceptible to protease digestion, although SDS-PAGE results (Fig. 3.2B) suggest that the opposite may be the case. Support for the hypothesis that oxidation may protect HDL from protease digestion comes from a recent study by Burcham and Kuhan (Burcham & Kuhan 1997), which demonstrates that albumin oxidized using the lipid peroxidation product malondialdehyde is resistant to proteolysis by chymotrypsin.

It is also possible that some active apolipoprotein fragments may be present on tyrosylated HDL even after extended treatment with trypsin or after treatment with the broad-spectrum protease subtilisin. Further investigation of this problem could be performed using tricine gel electrophoresis of protease-treated tyrosylated HDL to separate the low molecular weight smear seen using SDS-PAGE into distinct bands (Schagger & Jagow 1987).

Chapter 4: Delipidated Apolipoproteins and Reconstituted HDL Particles

4.1 Introduction

As outlined in Chapter 3, the results from protease treatment of HDL before or after tyrosyl radical-mediated oxidation were not sufficient to discern whether enhancement of cholesterol efflux by tyrosylated HDL is due to modification of HDL protein or lipid components. Therefore, it was necessary to assess directly the role of the apolipoprotein fraction of tyrosyl radical-oxidized HDL. In the following set of experiments, the apolipoprotein fractions of HDL and Tyr-HDL were isolated by delipidation, and combined with phosphatidylcholine and cholesterol or with the whole lipid extract of HDL to form reconstituted HDL particles (r-HDL) using the cholate dialysis method. Delipidated apo A-I and apo A-II were also oxidized with tyrosyl radical and used to prepare r-HDL. The abilities of both delipidated apolipoproteins and r-HDL to stimulate cholesterol efflux were assessed in cell culture experiments.

As was discussed in section 1.2.2, the ability of delipidated apolipoproteins to act as promoters of cholesterol efflux has previously been demonstrated using lipid-free apolipoproteins A-I, A-II, A-IV, and E, and insect apolipophorin III (Bielicki *et al.* 1992; Hara & Yokoyama 1991; Oram & Yokoyama 1996; vonEckardstein 1996).

Reconstituted HDL particles are usually prepared by either the cholate dialysis method (Jonas 1986; Matz & Jonas 1982) or by the sonication method (Pittman *et al.* 1987; Ritter

& Scanu 1977; Schonfeld *et al.* 1975), and r-HDL prepared using both these methods has been used to study cellular cholesterol efflux (Barkia *et al.* 1991; Bernini *et al.* 1996; Davidson *et al.* 1994; Davidson *et al.* 1995; Gillotte *et al.* 1996; vonEckardstein *et al.* 1993; Westman *et al.* 1993; Zhao *et al.* 1996a; Zhao *et al.* 1996b). Particles prepared by cholate dialysis using phosphatidylcholine and cholesterol typically are discoidal in shape (Matz & Jonas 1982). The cholate dialysis method has not previously been used by other investigators to incorporate neutral lipids into r-HDL; however, r-HDL prepared by this method have been treated with purified LCAT to form spherical particles with a cholesteryl ester core (Jonas 1986). The sonication method has previously been used to incorporate both phospholipids and neutral lipids into r-HDL (Pittman *et al.* 1987; Ritter & Scanu 1977; Schonfeld *et al.* 1975). In our experiments, we chose not to prepare r-HDL using sonication due to concerns about the stability of the particles (Jonas 1986) and the possibility of exposing the r-HDL to transition metal ions from the sonication probe.

If the apolipoprotein fraction of tyrosylated HDL were responsible for its increased ability to stimulate cholesterol efflux, we expected that tyrosyl radical-oxidized apolipoproteins would deplete intracellular cholesterol to a greater extent than unoxidized apolipoproteins.

4.2 Results

4.2.1 Delipidated apolipoprotein A-I and A-II

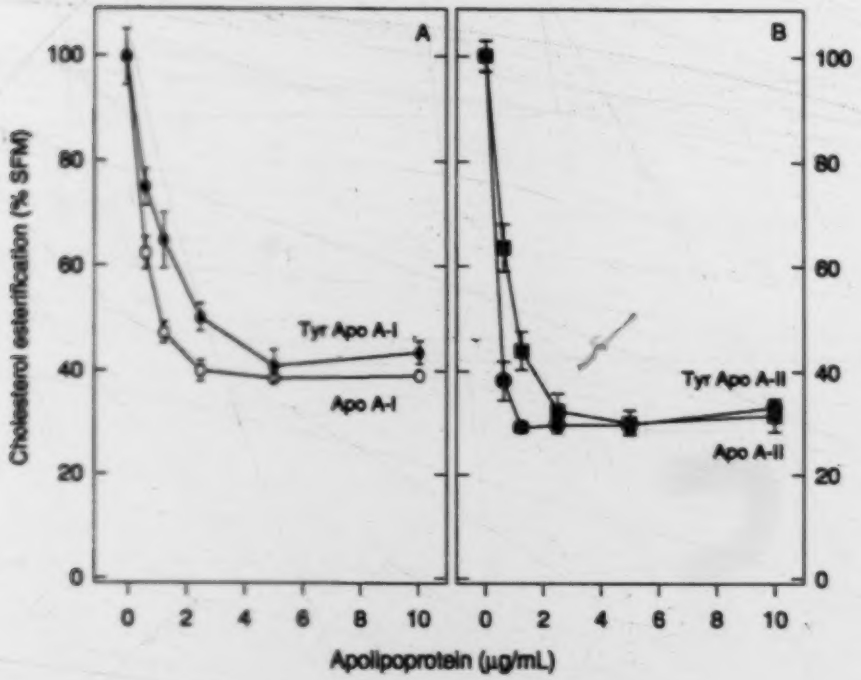


Figure 4.1. Cholesterol esterification in fibroblasts incubated with lipid-free apo A-I and apo A-II. Cholesterol-loaded human fibroblasts were incubated with the indicated concentration of control or tyrosylated apo A-I (A) or apo A-II (B) for 16 h. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Results are expressed as percentage of the serum-free medium value. Values are the mean +/- SD of 4 determinations.

To assess the effect of tyrosyl radical-mediated oxidation of the apolipoproteins of HDL directly, I initially explored the effects of oxidation on delipidated apo A-I and apo A-II purified by column chromatography (section 2.2.4). Oxidation of delipidated apo A-I by tyrosyl radical (section 2.2.5) produced apo A-I cross-links similar to those seen with oxidation of intact HDL. However, tyrosylated lipid-free apo A-I was no more potent

than control apo A-I at promoting cellular cholesterol efflux (Figure 4.1A). Similar results were seen upon tyrosyl radical-mediated oxidation of lipid free apo A-II (Figure 4.1B).

4.2.2 Apolipoproteins isolated from tyrosyl radical-oxidized HDL, delipidated or as discoidal reconstituted HDL particles

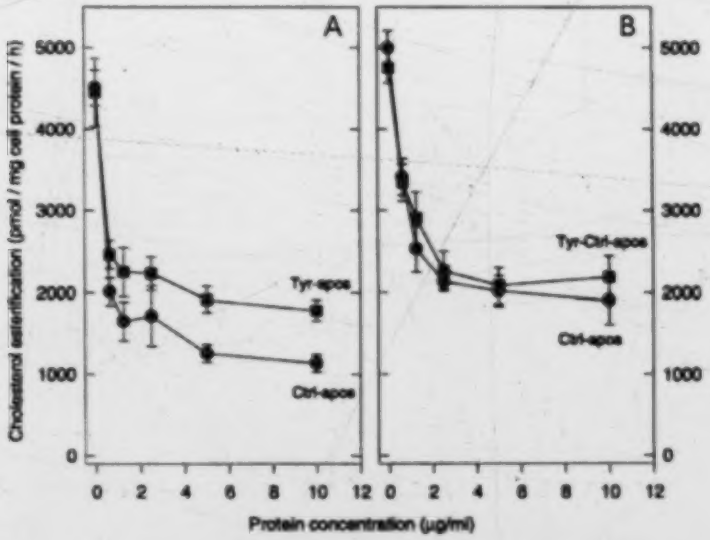


Figure 4.2. Cholesterol esterification in fibroblasts incubated with delipidated HDL apolipoproteins. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of delipidated apolipoproteins from control HDL (Ctrl-apos) or tyrosylated HDL (Tyr-apos)(**A**), or with tyrosylated apolipoproteins from control HDL (Tyr-Ctrl-apos)(**B**). The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Values are the mean +/- SD of 4 determinations and are representative of two similar experiments.

Next, I isolated the apolipoproteins of HDL or tyrosylated HDL by delipidation (section 2.2.6.2), and assessed their ability to deplete intracellular cholesterol using the cholesterol esterification assay (section 2.2.2). Delipidated apolipoproteins from tyrosylated HDL (tyr-apos) did not stimulate intracellular cholesterol efflux more than delipidated apolipoproteins from control HDL (ctrl-apos)(Figure 4.2); furthermore, tyrosylation of isolated ctrl-apos did not increase their ability to stimulate cholesterol efflux (Figure 4.2).

Although both the control and tyrosylated apolipoproteins are excellent promoters of cholesterol efflux, the possibility exists that tyrosylated apolipoproteins need to be associated with lipid to enhance cholesterol efflux, or need to be associated with lipid during the tyrosylation process to be "activated". To distinguish between these possibilities, I reconstituted ctrl-apos or tyr-apos with phosphatidylcholine and cholesterol using the cholate dialysis method (section 2.2.6.3). Characterization of reconstituted HDL (r-HDL) produced by this method in other laboratories has shown that the particles have a discoidal shape (Matz & Jonas 1982); this was confirmed by transmission electron microscopy in collaboration with Dr. Ming Chen (Appendix 3).

When ctrl-apos and tyr-apos were incorporated into discoidal r-HDL, the tyr-r-HDL showed no difference in its ability to deplete cells of cholesterol compared to ctrl-r-HDL (Figure 4.3). As with the delipidated apolipoproteins, tyrosylation of discoidal ctrl-r-HDL did not increase its ability to stimulate cholesterol efflux (Figure 4.3), despite showing typical cross-links on SDS-PAGE (Figure 4.4).

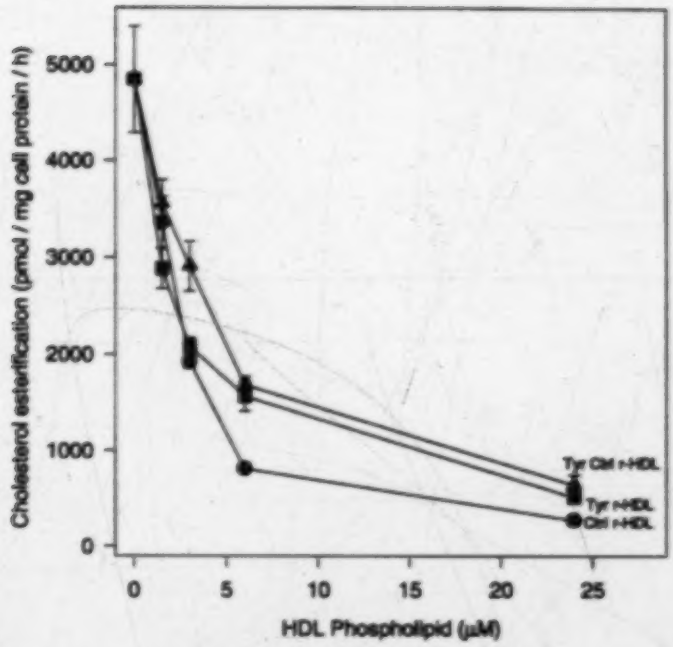


Figure 4.3. Cholesterol esterification in fibroblasts incubated with reconstituted HDL containing delipidated HDL apolipoproteins. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of reconstituted HDL containing apolipoproteins from control (Ctrl r-HDL) or tyrosylated HDL (Tyr r-HDL), or with tyrosylated Ctrl r-HDL (Tyr Ctrl r-HDL). The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Values are the mean +/- SD of 4 determinations and are representative of two similar experiments.

To rule out the possibility that the lack of difference between the r-HDL particles resulted from the presence of excess lipid-free apolipoproteins, the r-HDL preparations were ultracentrifuged to remove any excess protein. However, ultracentrifugation did not

result in a difference between stimulation of cholesterol efflux by ctrl-r-HDL and tyr-r-HDL (Figure 4.5).

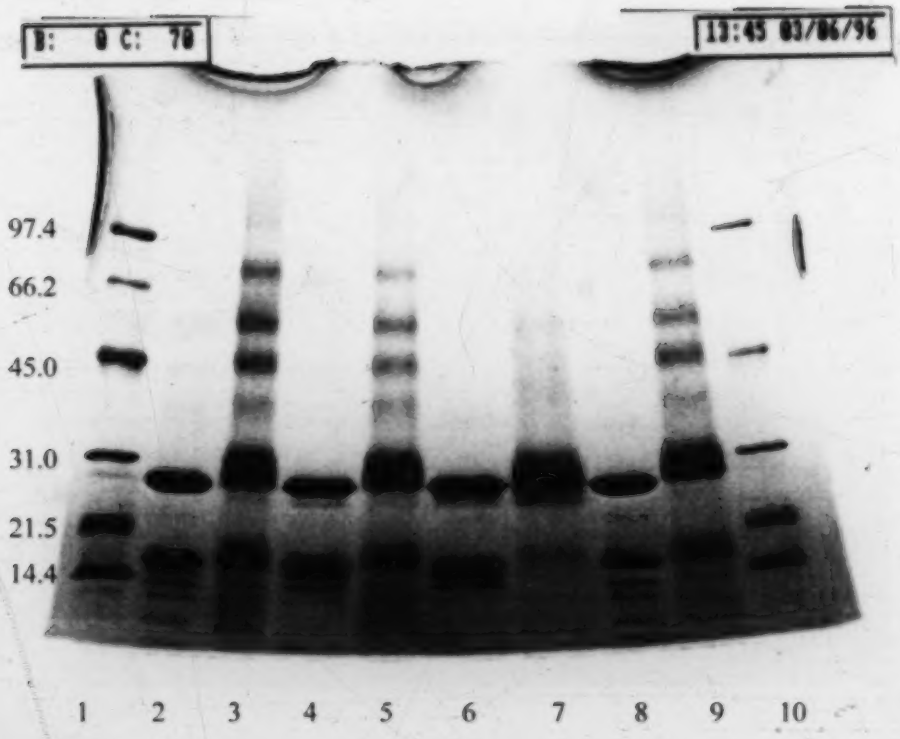


Figure 4.4. SDS-PAGE of delipidated apolipoproteins and reconstituted HDL particles. Lanes:1 and 10, low molecular weight markers; 2, delipidated apolipoproteins from HDL; 3, delipidated apolipoproteins from tyrosylated HDL; 4, discoidal rHDLprepared using 2 (ctrl-rHDL); 5, discoidal rHDL prepared using 3 (tyr-rHDL); 6, ctrl-rHDL which has been incubated in the absence of tyrosyl radical at 37 °C for 18h; 7, ctrl-rHDL which has been incubated with tyrosyl radical at 37 °C for 18h; 8, HDL_3 which has been incubated in the absence of tyrosyl radical at 37 °C for 18h (ctrl-HDL); 9, HDL_3 which has been incubated with tyrosyl radical at 37 °C for 18h (tyr-HDL). Molecular mass (kDa) is indicated.

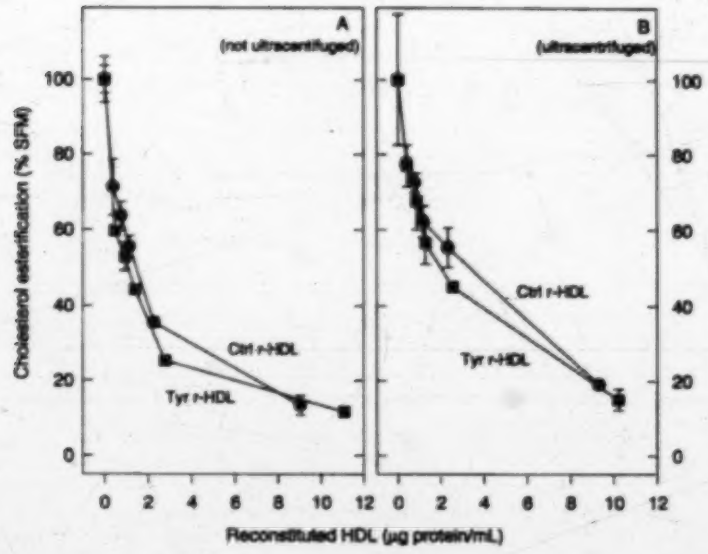


Figure 4.5. Cholesterol esterification in fibroblasts incubated with reconstituted HDL containing delipidated HDL apolipoproteins. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of reconstituted HDL containing apolipoproteins from control (Ctrl r-HDL) or tyrosylated HDL (Tyr r-HDL). The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. A, reconstituted particles; B, reconstituted particles which had been ultracentrifuged (18 h, 99000 rpm, 4 °C, in TL 100.3 rotor, at d = 1.21 g/mL). Values are the mean +/- SD of 4 determinations and are representative of two similar experiments.

4.2.3 Apolipoproteins isolated from tyrosyl radical-oxidized HDL, incorporated into spherical reconstituted HDL particles

Taken together, the results of the experiments using the discoidal r-HDL suggested that apolipoproteins alone did not mediate the stimulation of cholesterol efflux by tyrosylated HDL. However, the conformation of apolipoproteins on the surface of discoidal r-HDL may differ substantially from that on a spherical particle, and the possibility remained that a difference in the activity of control and tyrosylated apolipoproteins might be seen if spherical r-HDL were used in place of discoidal r-HDL in these experiments. Indeed, Dr. Wen-Qi Wang's preliminary experiments using r-HDL produced by cholate dialysis using the whole lipid extract from HDL in place of commercial PC and FC (Appendix 1) suggested that such a difference became apparent when the apolipoproteins were presented to cells on the surface of spherical particles. Therefore the above experiments were repeated using the whole lipid extract of control or Tyr-HDL to make spherical r-HDL particles, as described in section 2.2.6.3. Incorporation of neutral lipids was confirmed by measurements of chemical composition (Appendix 3); furthermore, transmission electron microscopy confirmed that the particles appear spherical (Appendix 3).

In contrast to our results using discoidal r-HDL, those r-HDL particles containing delipidated apolipoproteins from tyrosylated HDL had a greater ability to deplete cellular cholesterol than those containing apolipoproteins isolated from control HDL (Figure 4.6). The particles containing tyrosylated lipids and control apolipoproteins showed 50-60% of the activity of particles containing tyrosylated apolipoproteins, in contrast to the preliminary results using corresponding particles shown in Appendix 1. This discrepancy was felt to result from trace protein contamination of the lipid extracts in some

experiments.

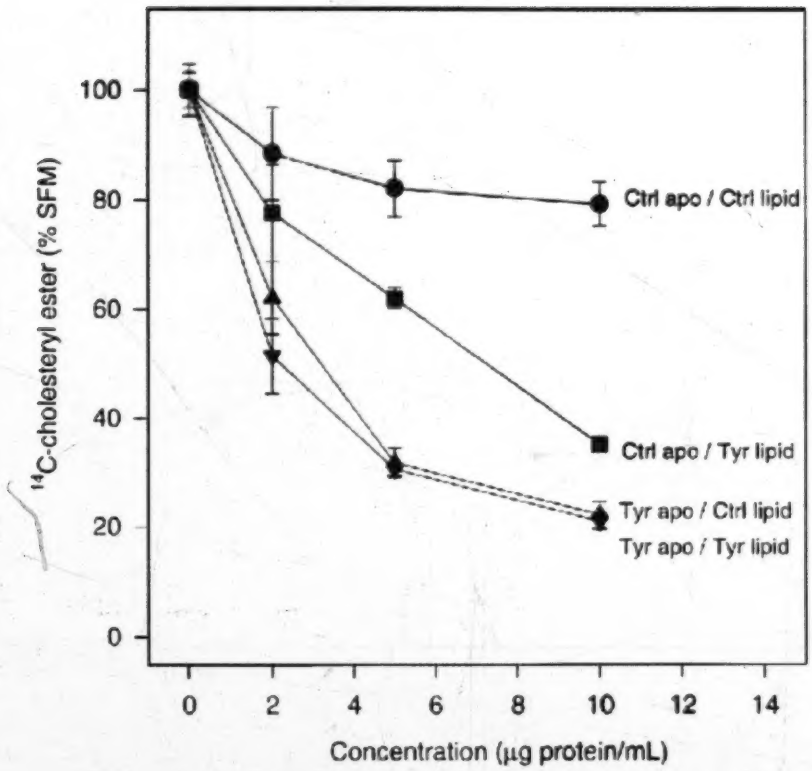


Figure 4.6. Cholesterol esterification in fibroblasts incubated with reconstituted HDL containing delipidated HDL apolipoproteins and whole HDL lipids. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of r-HDL containing the indicated apolipoprotein and lipid fractions. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: Ctrl apo, apolipoproteins isolated from HDL; Tyr apo, apolipoproteins isolated from tyrosylated HDL; Ctrl lipid, whole lipid extract from HDL; Tyr lipid, whole lipid extract from tyrosylated HDL. Values are the mean +/- SD of 4 determinations and are representative of three similar experiments.

4.2.4 Purified apolipoproteins A-I and A-II, incorporated into spherical reconstituted HDL particles

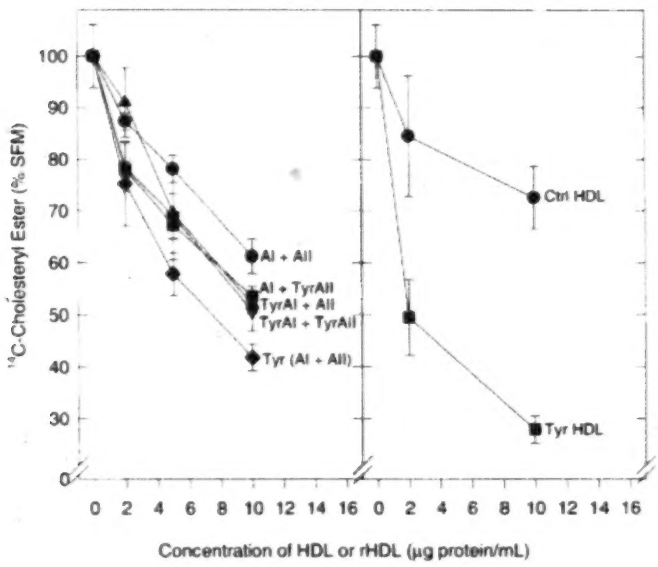


Figure 4.7. Cholesterol esterification in fibroblasts incubated with reconstituted HDL containing a 2:1 (mol:mol) mixture of apo A-I and apo $(A\text{-}II)_2$, and whole HDL lipid. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of r-HDL containing the indicated apolipoproteins and the whole lipid extract of HDL. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: Ctrl HDL, control HDL; Tyr HDL, tyrosylated HDL; AI + AII, r-HDL containing apo A-I and apo $(A\text{-}II)_2$; AI + TyrAII, r-HDL containing apo A-I and tyrosylated apo $(A\text{-}II)_2$; TyrAI + AII, r-HDL containing tyrosylated apo A-I and apo $(A\text{-}II)_2$; TyrAI + TyrAII, r-HDL containing apo A-I and apo $(A\text{-}II)_2$ which were tyrosylated separately; Tyr(AI + AII), r-HDL containing apo A-I and apo $(A\text{-}II)_2$ which were tyrosylated as a mixture. Values are the mean +/- SD of 4 determinations.

To further elucidate the role of apolipoproteins in enhancement of cholesterol efflux by tyrosylated HDL, and to determine if the formation of crosslinks between apo A-I and apo A-II was required, delipidated apo A-I and apo A-II were used to prepare reconstituted particles. Delipidated apo A-I and apo $(A\text{-}II)_2$ were tyrosylated separately or as a mixture (molar ratio 2: 1, A-I/A-II; section 2.2.5), then used in cholesterol efflux experiments as spherical r-HDL (Figure 4.7; Appendix 4).

Spherical r-HDL prepared using a tyrosyl radical-oxidized mixture of apo A-I and apo A-II showed a significantly increased ability to stimulate cholesterol efflux compared with r-HDL prepared using unoxidized apolipoproteins (Figure 4.7, Appendix 4). Spherical r-HDL that contain apo A-I or apo A-II or apolipoproteins which were exposed to tyrosyl radical separately showed a smaller increase in cholesterol removal.

4.3 Summary

These results represent the first strong evidence that the apolipoprotein fraction of tyrosylated HDL is responsible for its increased ability to stimulate cholesterol efflux. They also suggest that apolipoprotein conformation may be important, since the difference in activity between unoxidized and tyrosylated apolipoproteins was only apparent when they were presented on the surface of spherical r-HDL particles. Notably, the results of experiments using tyrosyl radical-oxidized mixtures of apo A-I and apo A-II

suggested that an AI-AII crosslinked species might be involved in the stimulation of cholesterol efflux by tyrosylated HDL.

Chapter 5: HDL Particles with Defined Apolipoprotein Compositions

5.1 Introduction

Taken together, the results of the reconstitution experiments discussed in Chapter 4 demonstrated that the apolipoprotein fraction of tyrosylated HDL is responsible for its increased ability to stimulate cholesterol efflux, and suggested that an apo AI-AII crosslinked species might be responsible for this enhanced effect. To confirm if the generation of apo AI-AII crosslinks is necessary for the enhancement of cholesterol efflux by tyrosylated HDL, two experimental methods were used to prepare HDL populations containing apo A-I only, apo A-II only, or both apo A-I and apo A-II, for use in oxidation experiments.

To generate particles containing only apo A-II, HDL_3 was incubated with an excess of apo A-II to displace all the apo A-I on its surface. This method has been previously used to displace apo A-I from canine HDL (Edelstein *et al.* 1982; Lagocki & Scanu 1980), reconstituted HDL (Rye 1990), total human HDL (van Tornout *et al.* 1981), and human HDL_3 (Lagrost *et al.* 1995; Lagrost *et al.* 1994; Vadiveloo & Fidge 1990).

To generate particles containing only apo A-I and particles containing both apo A-I and apo A-II, HDL_3 was subjected to immunoaffinity column chromatography. This method was used previously to demonstrate that human HDL can be divided into two main populations of particles: those containing only apo A-I, termed LpAI; and those

containing both apo A-I and apo A-II, termed LpAI/AII (Cheung & Albers 1984). Immunopurified HDL subfractions have been shown to differ in their abilities to react with plasma remodeling enzymes (Duverger *et al.* 1994; Lagrost *et al.* 1994; Mowri *et al.* 1992; Nichols *et al.* 1989) and their ability to stimulate cholesterol efflux from Ob1771 mouse adipose cells (Barbaras *et al.* 1987; Barkia *et al.* 1991) but not from hepatoma, fibroblast, smooth muscle, or endothelial cell lines (Johnson *et al.* 1991a; Oikawa *et al.* 1993).

If the generation of apo AI-AII crosslinks were necessary for the enhancement of cholesterol efflux by tyrosylated HDL, we expected that, after oxidation by tyrosyl radical, only those HDL populations which contained both apo A-I and apo A-II would reproduce the ability of tyrosylated HDL to enhance cholesterol efflux.

5.2 Results

5.2.1 Apolipoprotein AII-only HDL prepared by displacement

To confirm whether an apo AI-AII crosslinked species was necessary to enhance cholesterol efflux by tyrosylated HDL, we incubated HDL_3 with apo A-II at a protein ratio of 1 mg HDL:6 mg apo A-II, as described in section 2.2.7. The apo A-II-enriched HDL was ultracentrifuged to remove excess lipid-free apolipoproteins, then oxidized with tyrosyl radical (section 2.2.5). The abilities of the particles to enhance cholesterol efflux were compared using the cholesterol esterification assay (section 2.2.2).

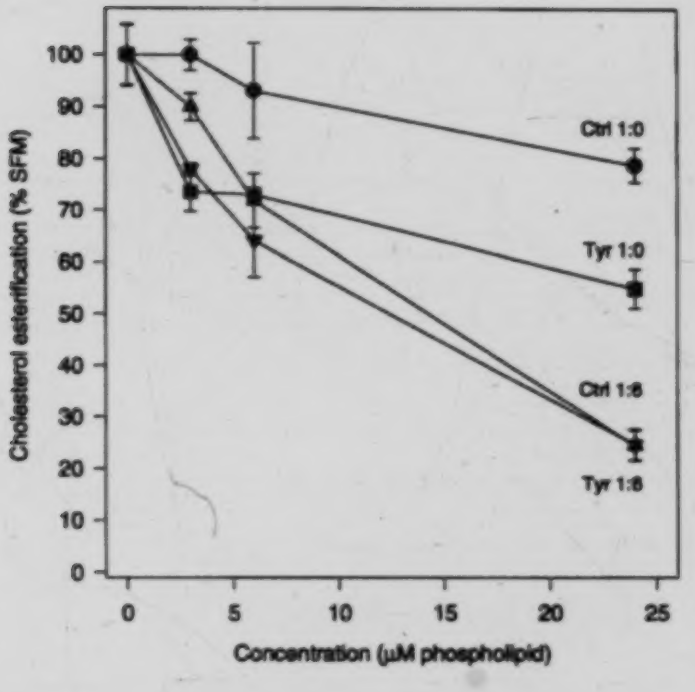


Figure 5.1. Cholesterol esterification in fibroblasts incubated with apo A-II-only HDL prepared by displacement of apo A-I with apo A-II. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of HDL. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: Ctrl 1:0, HDL incubated in the absence of apo A-II; Tyr 1:0, HDL incubated in the absence of apo A-II, then oxidized with tyrosyl radical; Ctrl 1:6, HDL incubated in the presence of apo A-II (1 mg HDL : 6 mg apo A-II, 2 h at 37 °C); Tyr 1:6, HDL incubated in the presence of apo A-II, then oxidized with tyrosyl radical. Values are the mean +/- SD of 3 determinations and are representative of three similar experiments. Results are expressed as percentage of the serum free medium value.

As shown in Figure 5.1, the tyrosylated AII-only HDL did not differ significantly from control in its ability to deplete cellular cholesterol. Both control and tyrosylated AII-only HDL had increased ability to stimulate cholesterol efflux compared to HDL and tyrosylated HDL. SDS-PAGE demonstrated that the AII-only HDL did not contain any residual apo A-I, and that tyrosylated A-II-only HDL did not contain any apo AI-AII crosslinked species (data not shown).

5.2.2 Immunoaffinity-purified HDL subfractions

Next, the ability of HDL subfractions purified by immunoaffinity chromatography (section 2.2.8) to promote cholesterol efflux was tested. Tyrosyl radical oxidation of LpAI/AII (Figure 5.2A), but not LpAI (Figure 5.2B), resulted in an enhanced ability of these particles to stimulate cholesterol efflux.

As shown in Figure 5.3, the LpAI did not contain any apoAII. Tyrosyl radical-mediated oxidation of both LpAI and LpAI/AII gave a number of high molecular weight crosslinks similar to those we see in TyrHDL. Those crosslinked apolipoprotein species which have been identified based on the results of Western blot analysis (Appendix 5) are: an apolipoprotein A-I/A-II heterodimer (37 kDa); an apolipoprotein A-I(A-II)$_2$ heterodimer (46 kDa); an apo A-I dimer (56 kDa), and an apo A-I trimer (84 kDa). Any higher molecular weight crosslinks have not been identified to date.

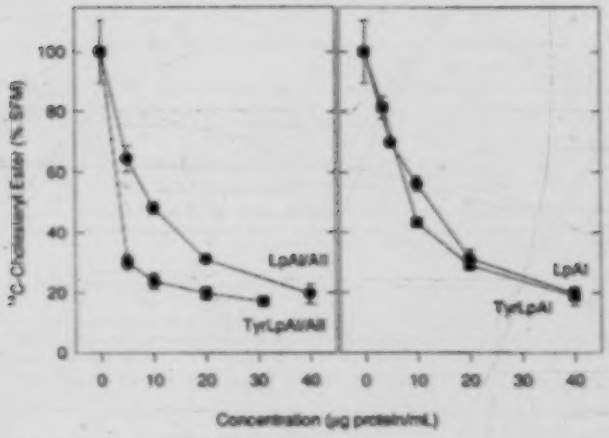


Figure 5.2. Cholesterol esterification in fibroblasts incubated with immunoaffinity-purified HDL subfractions. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of HDL. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: LpAI, HDL containing only apo A-I; TyrLpAI, tyrosyl radical-oxidized LpAI; LpAI/AII, HDL containing both apo A-I and apo A-II; TyrLpAI/AII, tyrosyl radical-oxidized LpAI/AII. Values are the mean +/- SD of 3 determinations and are representative of two similar experiments. Results are expressed as percentage of the serum free medium value.

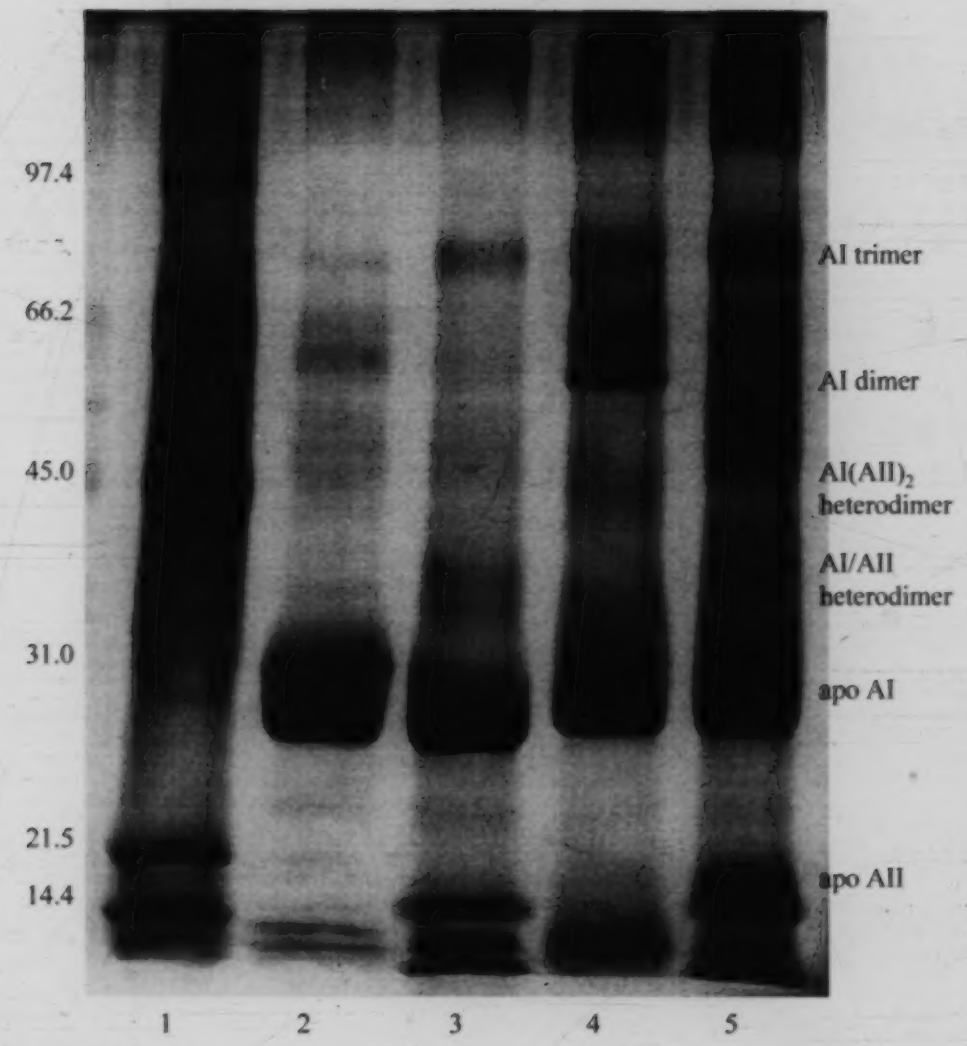


Figure 5.3. SDS-PAGE of immunoaffinity-purified HDL subfractions. Lanes: 1, low molecular weight markers; 2, LpAI; 3, LpAI/AII; 4, tyrosylated LpAI; 5, tyrosylated LpAI/AII. Molecular mass (kDa) and identities of crosslinks based on immunoblot results (Appendix 5) are indicated.

5.3 Summary

Taken together, these results further suggested that the enhanced cholesterol efflux by tyrosylated HDL is primarily due to formation of a unique AI-AII crosslinked species.

An alternative explanation for the results shown in Figure 5.1 is that displacement of apo A-I by apo A-II results in an excess of free apolipoproteins, or the creation of apo A-I-containing lipoprotein particles, which are not removed by the ultracentrifugation step. Some investigators use gel filtration chromatography in addition to ultracentrifugation to ensure that lipid-free apolipoproteins have been removed (Edelstein *et al.* 1982). Furthermore, Castro has reported that the displacement of apo A-I from native or reconstituted HDL by apo A-II results in the formation of apo A-I-containing pre-β HDL in addition to AII-only HDL particles (Castro 1997). Further investigations using apo A-II to displace apo A-I from the surface of HDL may benefit from an additional gel filtration step and modification of the ultracentrifugation step to separate AII-only HDL from any lipid-free apolipoproteins or pre-β HDL particles.

Chapter 6: Purification of Crosslinked Apolipoproteins from Tyrosylated HDL

6.1 Introduction

Taken together, the results of the experiments discussed in Chapter 5 suggest that the enhanced cholesterol efflux by HDL oxidized by tyrosyl radical is due to formation of an apo AI-AII crosslinked species. To confirm this hypothesis, it was necessary to purify each of the crosslinked apolipoprotein species generated during tyrosyl radical-mediated oxidation of HDL. The purified apolipoprotein species could then be used to prepare reconstituted HDL particles, and test their ability to stimulate cholesterol efflux from cultured cells. We expected that one or more crosslinked species containing both apo A-I and apo A-II would have a greater ability to promote efflux of intracellular cholesterol if crosslinking of apo A-I and apo A-II were responsible for the enhancement of cholesterol efflux by tyrosylated HDL.

6.2 Results

6.2.1 Purification of crosslinked apolipoprotein species

A number of different methods were used in attempts to purify the crosslinked apolipoprotein species of tyrosyl radical-oxidized HDL. These methods included reversed phase high performance liquid chromatography (HPLC), fast phase liquid chromatography (FPLC) using an anion exchange column, size exclusion HPLC, and preparative electrophoresis.

6.2.1.1 Reversed phase high performance liquid chromatography

In reversed phase HPLC, molecules are separated based on partitioning between two liquid phases: the hydrophobic stationary phase, which is chemically bonded to the solid support, and the polar mobile phase, which flows freely through the column. The more hydrophobic a molecule is, the longer it will be retained on a reversed phase column.

The delipidated apolipoprotein fraction of tyrosylated HDL (section 2.2.6.2) was dissolved in buffer and was loaded onto the reversed phase HPLC column with the assistance of Dr. Paul Weers. The proteins were then eluted with a linear gradient of 0 to 80% acetonitrile, as described in section 2.2.9.1. As Figure 6.1 shows, distinct peaks were seen for the apo Cs, apo A-I, and apo A-II, but crosslinked species eluted as a broad shoulder.

Figure 6.1 (page 79) Reversed phase HPLC of delipidated apolipoproteins from tyrosylated HDL. 185 μg was loaded onto the Zorbax RX-C8 column, and eluted using a linear gradient from 0 to 80% acetonitrile as described in section 2.2.9.1. Protein peaks were identified by comparison with controls (data not shown) and published values: A, apo Cs; B, apo A-I; C, apo A-II; D, crosslinked species.

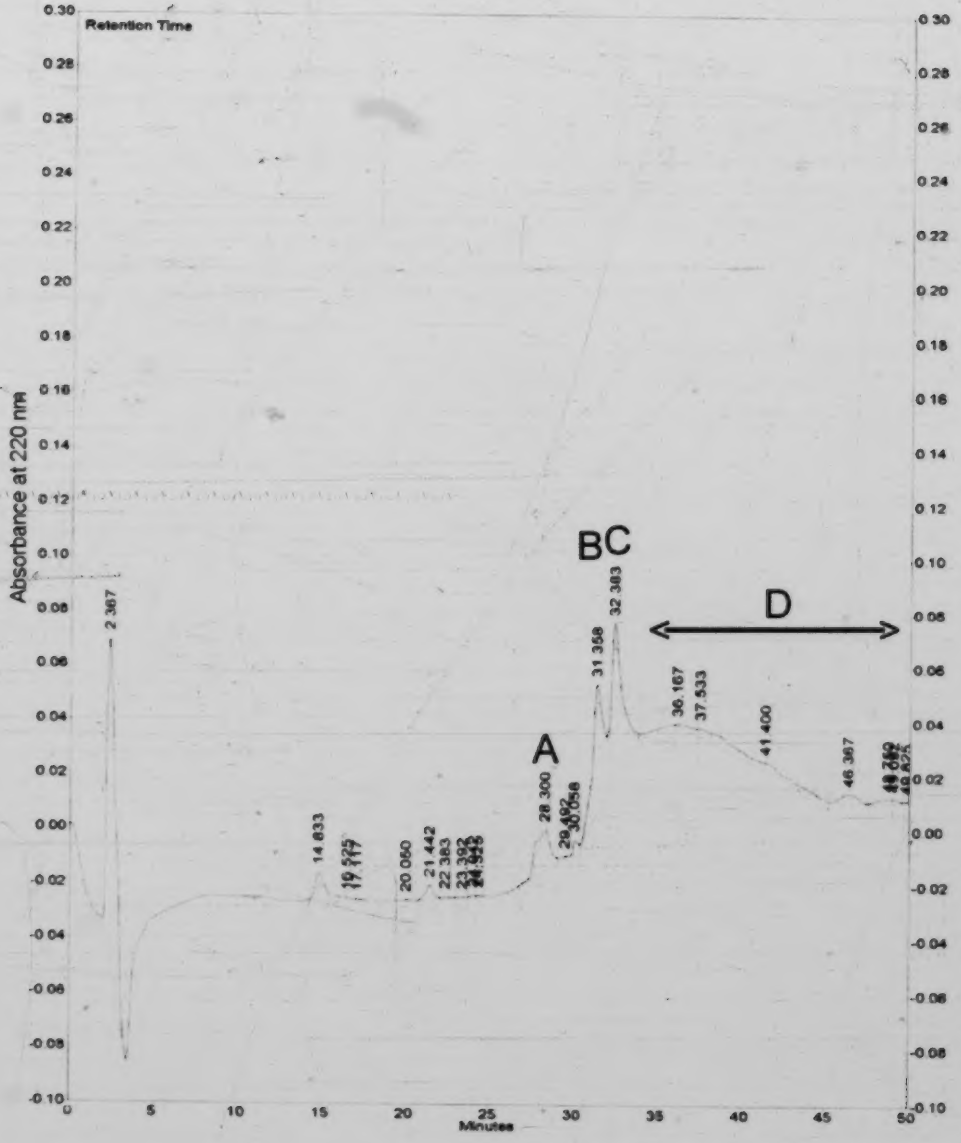

Retention Time
Absorbance at 220 nm
Minutes
A
BC
D
2.367
14.833
20.050
21.442
28.300
31.358
32.383
36.167
37.533
41.400
46.367

Figure 6.1

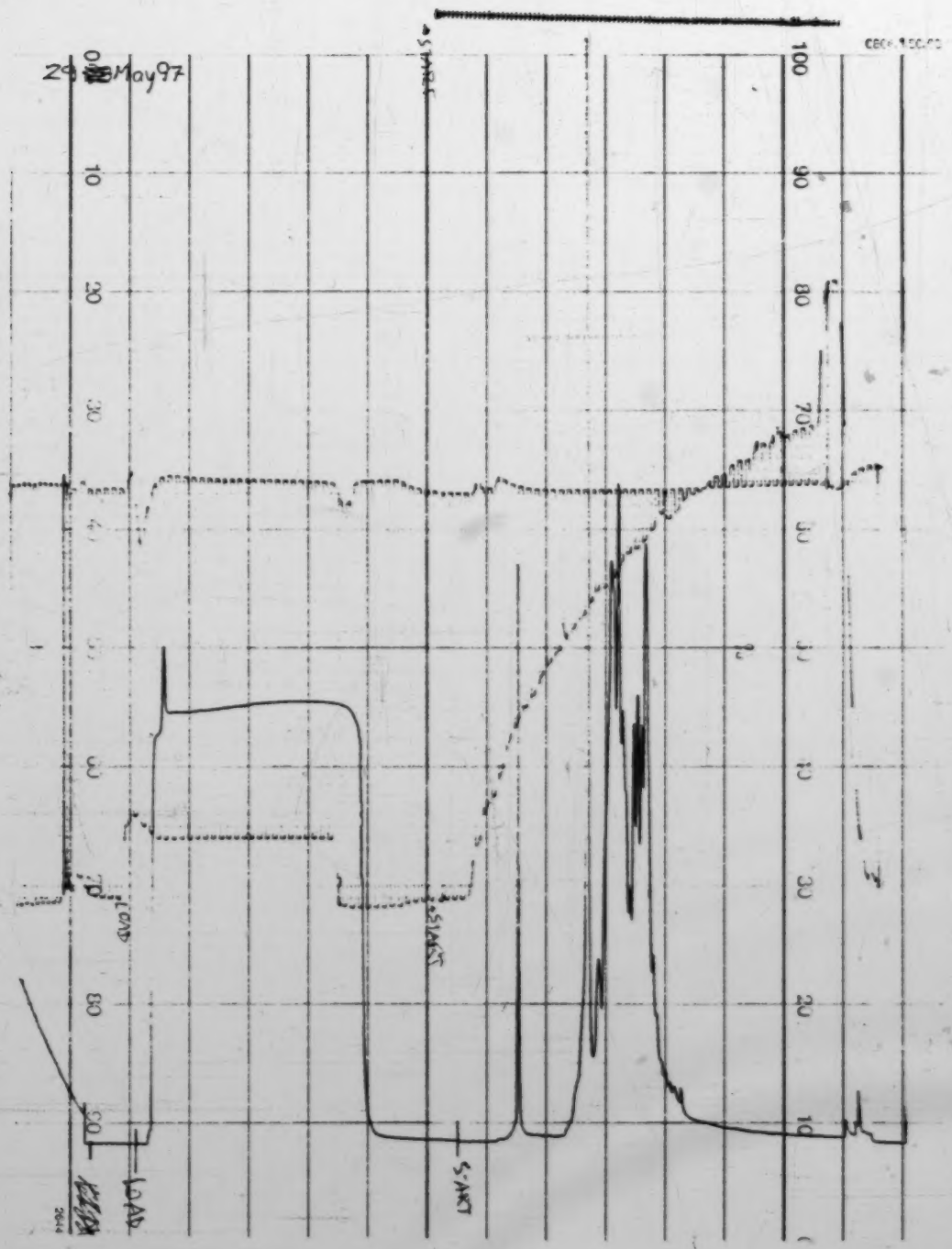
6.2.1.2 Fast phase liquid chromatography using an anion exchange column

Fast phase liquid chromatography (FPLC) was developed by Pharmacia Biotech to provide a purification system that bridges the gap between standard and high performance liquid chromatography and allows the use of many different chromatographic techniques. In anion exchange chomatography, molecules are separated based on their charge; the greater the negative charge of the molecule, the higher the retention time as a result of ionic interactions with cationic sites covalently attached to the column support material. Proteins are eluted by increasing the ionic strength or changing the pH of the buffer. We used MonoQ, an anion exchange resin consisting of quaternary amine groups linked to a beaded hydrophilic support.

Figure 6.2. (page 81) Anion exchange FPLC of delipidated apolipoproteins from tyrosylated HDL. 14 mg of sample was loaded onto the Mono Q column, and eluted using a linear gradient from 0 to 20% 2M NaCl in the presence of 8M urea as described in section 2.2.9.2. Flow rate was 0.8 mL/min; chart recorder was set for 0.16 cm/mL. The Y-axis is absorbance at 280 nm (blue) and pH (red).

With the assistance of Dr. David Waggonner, the delipidated apolipoprotein fraction of tyrosylated HDL was loaded onto the MonoQ FPLC column, and the proteins were eluted using a linear gradient of NaCl in the presence of 8M urea (Figure 6.2). Aliquots from key fractions were used for SDS polyacrylamide gel electrophoresis to assess the extent of separatation of the crosslinked species. As shown in Figure 6.3, the separation of the crosslinked species was poor even in the presence of urea.

Figure 6.2



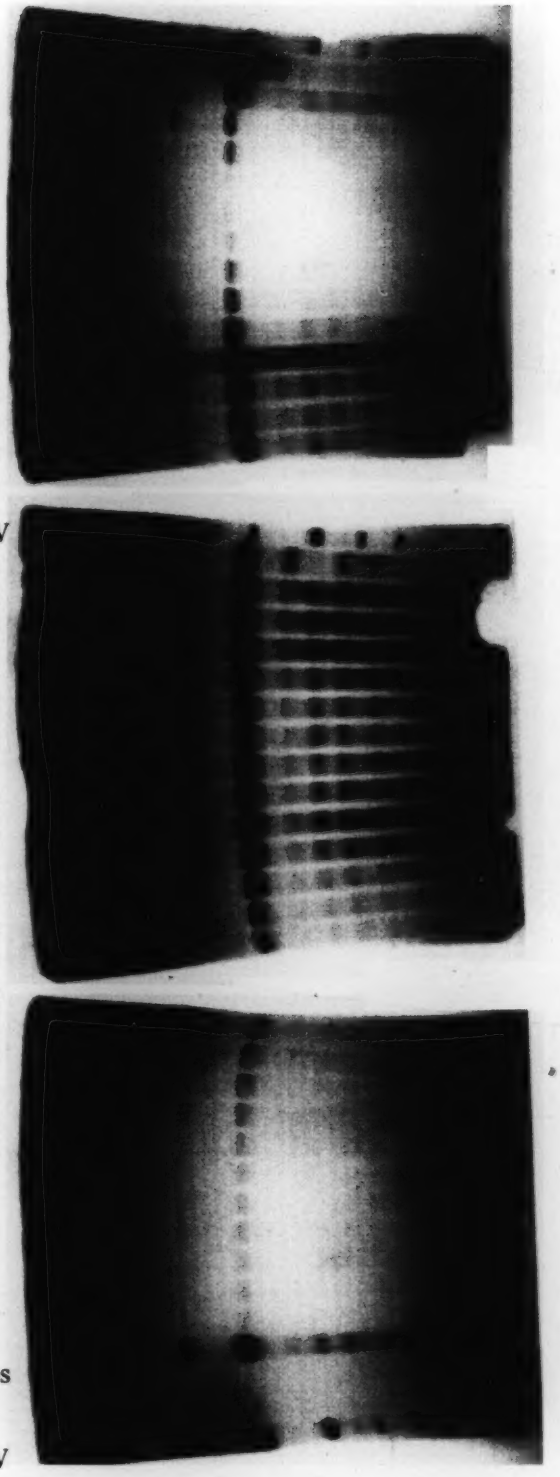
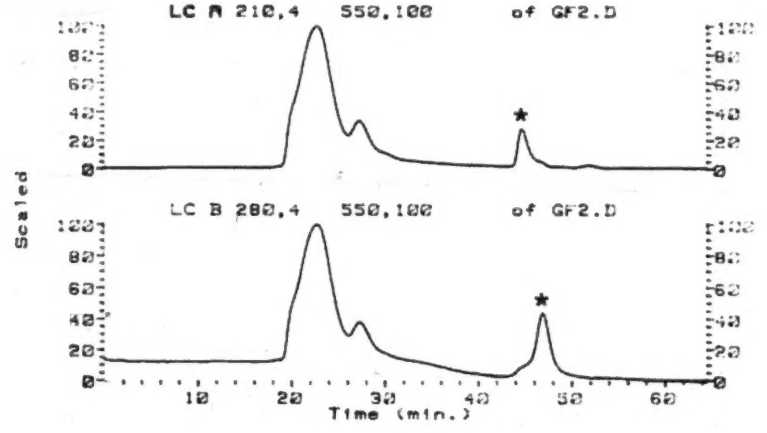


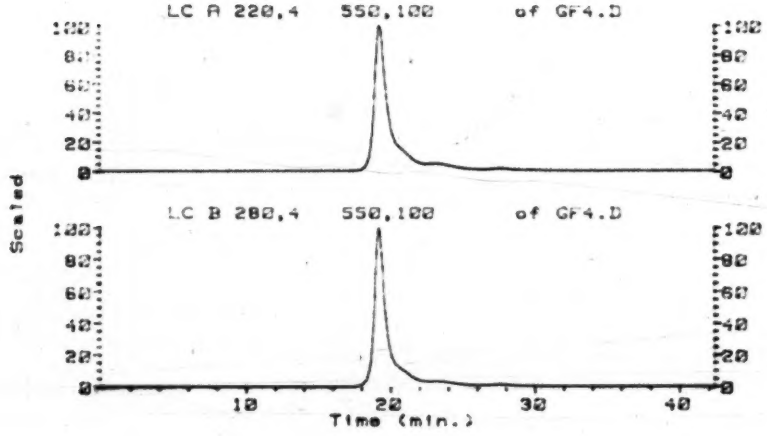
Figure 6.3. SDS-PAGE of fractions collected during anion-exchange FPLC of delipidated apolipoproteins from tyrosylated HDL (Tyr apos). Molecular weight markers (LMW) are14.4 kDa, 21.5 kDa, 31.0 kDa, 45.0 kDa, 66.2 kDa, and 97.4 kDa. Fraction numbers are indicated.

Figure 6.4. Size exclusion HPLC of delipidated apolipoproteins from tyrosylated HDL. 14 mg (A) or 63 mg (B) of sample was loaded onto the Superdex 75 column, and eluted with 50 mM KH_2PO_4, 200 mM KCl, pH 7 in the absence (A) or presence (B) of 8M urea. Flow rate was 0.3 mL/min. The peak marked with * in panel A was identified as a biological contaminant based on its altered absorbance at 210 nm compared with 280 nm.

A



B



6.2.1.3 Size exclusion high performance liquid chromatography

In size exclusion HPLC, a column packed with a porous gel is used to separate molecules based on their molecular size in solution; larger molecules which cannot enter the pores of the column material are not retained and elute first. The delipidated apolipoprotein fraction of tyrosylated HDL was loaded on a Superdex 75 size exclusion column by Dr. Colin Mant and eluted in the absence (Figure 6.4A) or presence (Figure 6.4B) of 8M urea. These results indicate that size exclusion HPLC also did not separate the crosslinked apolipoprotein species into the individual protein peaks required to assess their relative abilities to promote cholesterol efflux.

6.2.1.4 Preparative polyacrylamide gel electrophoresis

In preparative polyacrylamide gel electrophoresis, as in SDS-PAGE, proteins are separated on the basis of size; generally speaking, the larger the protein, the more slowly it will move through pores in the polyacrylamide gel. We had previously been able to identify individual crosslinked apolipoprotein species in tyrosylated HDL by SDS-PAGE, but not by non-denaturing PAGE in the absence of SDS (data not shown). Tyrosyl radical-oxidized HDL was prepared as described in section 2.2.5, then delipidated and redissolved as described in section 2.2.6.2. The sample was then mixed with sample buffer, loaded onto the preparative gel, and resolved as described in section 2.2.9.3. Fractions were collected as they ran off the gel.

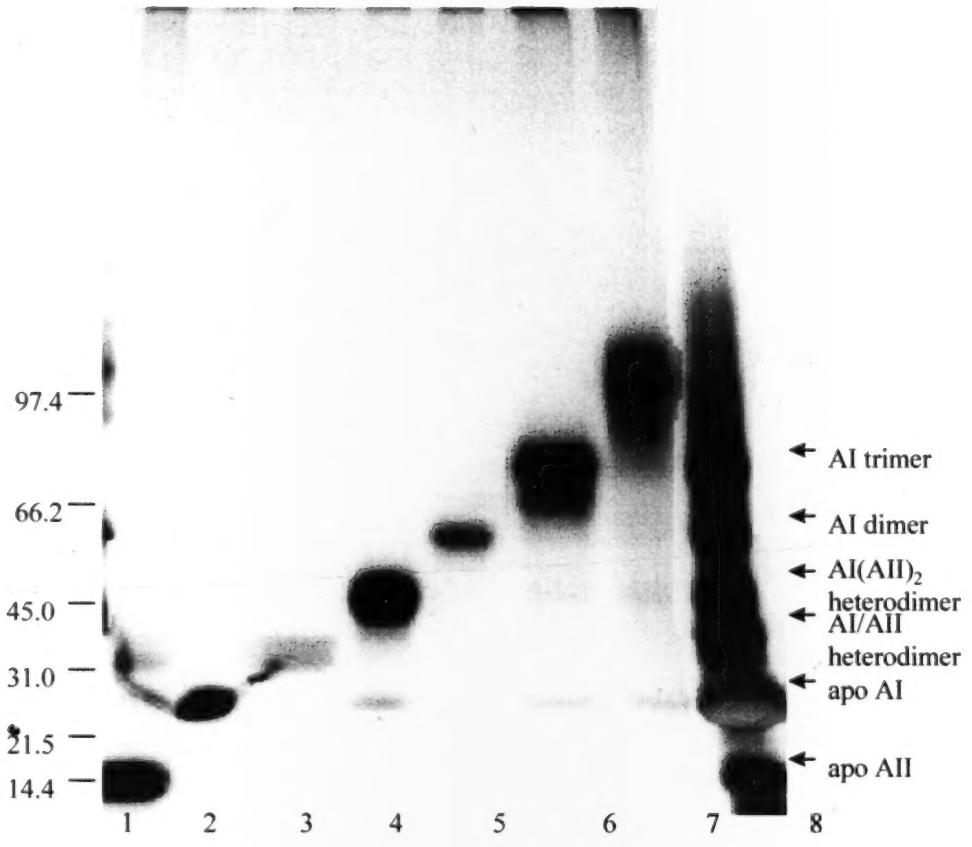


Figure 6.5. SDS-PAGE of pooled fractions from preparative gel electrophoresis. Lanes: 1, fractions 24-31, enriched in apo A-II; 2, fractions 42-54, enriched in apo A-I; 3, fractions 68-77, enriched in AI/AII heterodimer; 4, fractions 91-102, enriched in AI(AII)2 heterodimer; 5, fractions 126-136, enriched in apo A-I dimer; 6, fractions 160-181, enriched in apo A-I trimer; 7, fractions 269-305; 8, delipidated apolipoproteins from tyrosylated HDL. Molecular mass (kDa) and identities of crosslinks based on immunoblot results (Appendix 5) are indicated.

To assess the separatation of the crosslinked species, aliquots of every third fraction were resolved by SDS-PAGE (section 2.2.10.1). We found that the crosslinked species tended to elute as broad bands and most fractions contained traces of other species in addition to the predominant one. Based on the results of SDS-PAGE, the peak fractions were pooled, and their purity was assessed again by SDS-PAGE. As Figure 6.5 shows, each pooled sample was enriched in a single crosslinked apolipoprotein species.

6.2.2 Preparation of reconstituted HDL particles

SDS was removed from the purified crosslinked apolipoproteins by using AG-11 A8 ion retardation column chromatography, as described in section 2.2.9.4. The protein was then incorporated into spherical r-HDL with the lipid extract from HDL (section 2.2.6.3), and ultracentrifuged (section 2.2.6.4). The ability of each particle to stimulate cholesterol efflux was assessed using the cholesterol esterification assay described in section 2.2.2. Unfortunately, only enough material was obtained to treat cells with a single concentration of each species instead of a full range of concentrations.

As shown in Figure 6.6, r-HDL prepared using the apo AI-$(AII)_2$ crosslinked species (46 kDa) appeared to be best able to enhance cholesterol efflux. Subsequent experiments indicate that an apo AI-$AII_{monomer}$ crosslinked species is also capable of enhancing cholesterol efflux (Appendix 6).

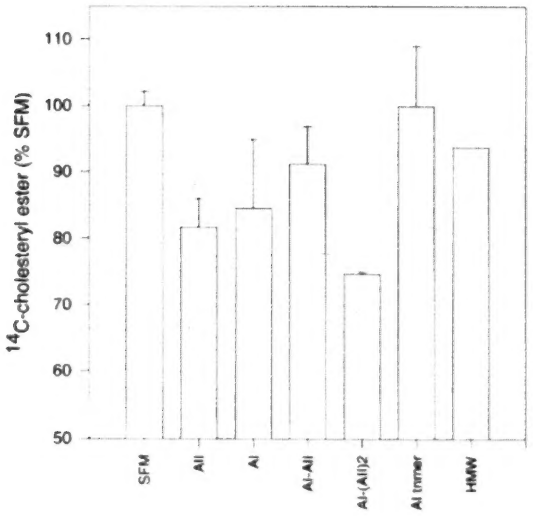


Figure 6.6. Cholesterol esterification in fibroblasts incubated with r-HDL particles enriched in tyrosylated apolipoprotein species partially purified by preparative electrophoresis. Cholesterol-loaded human fibroblasts were incubated for 16 h with 2 μg/mL medium of the indicated particle. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: SFM, serum-free medium; AII, r-HDL enriched in apo A-II; AI, r-HDL enriched in apo A-I; AI-AII, r-HDL enriched in the apo A-I/A-II heterodimer; AI-(AII)2, r-HDL enriched in the apo A-I(A-II)$_2$ heterodimer; AI trimer, r-HDL enriched in the apo A-I trimer; HMW, r-HDL enriched in a mixture of unidentified higher molecular weight crosslinked species. Values are the mean +/- SD of 3 determinations. Results are expressed as percentage of the serum free medium value.

6.3 Summary

Protein fractions enriched in specific crosslinked apolipoprotein species were prepared and presented to cells on the surface of spherical r-HDL particles to assess their relative

abilities to stimulate cholesterol efflux. The apo AI-$(AII)_2$ crosslinked species (46 kDa) stimulated cholesterol efflux to the greatest extent, strongly suggesting that an apo AI-AII crosslinked species is responsible for the enhancement of cholesterol efflux by tyrosylated HDL.

Purification of crosslinked apolipoprotein species using reverse phase HPLC, anion exchange FPLC, and size exclusion HPLC proved to be more difficult than was initially expected. Optimization of these methods might have resulted in enhanced separation of the apolipoprotein species; however, we instead chose preparative electrophoresis as a means of quickly and directly purifying the species.

Chapter 7: Summary, Concluding Remarks and Future Directions

Taken together, these studies provide strong evidence that the apolipoprotein fraction of tyrosyl radical-oxidized HDL is responsible for its increased ability to stimulate cholesterol efflux, and suggest that the component responsible for this enhanced effect is a crosslinked apo A-I - apo A-II species formed during oxidation by tyrosyl radical.

Protease digestion of lipoproteins initially was used to determine whether the enhancement of cellular cholesterol efflux by tyrosylated HDL is due to modification of HDL protein or lipid components. As was discussed in Chapter 3, these studies did not give conclusive results: either the enhancement of cholesterol efflux by tyrosylated HDL was not due to modification of its apolipoproteins, which we subsequently showed was not the case; or, some intact apolipoproteins or active apolipoprotein fragments were still present after protease treatment. Tyrosyl radical-mediated oxidation of HDL could render it less susceptible to protease digestion, as Burcham and Kuhan recently demonstrated is the case for malondialdehyde-modified albumin (Burcham & Kuhan 1997). However, SDS-PAGE of trypsin-treated tyrosylated HDL shows a smear of lower molecular-weight protein fragments (Figure 3.2B), which suggests that tyrosylated HDL may be more susceptible than control HDL to protease digestion. It is possible that some active apolipoprotein fragments are still present on tyrosylated HDL after protease treatment. If fragments of tyrosylated apolipoproteins are able to enhance cholesterol efflux, the minimum peptide length required for activity needs to be assessed. This could be tested

using tyrosylated apolipoprotein fragments purified by tricine gel electrophoresis (Schagger & Jagow 1987).

Next, the ability of delipidated HDL apolipoproteins to stimulate cellular cholesterol efflux was assessed. Control and tyrosyl radical-oxidized apolipoproteins showed no difference in their ability to stimulate efflux when presented to cells in their lipid-free form or as reconstituted particles; however, tyrosylated apolipoproteins were better able to enhance cholesterol removal than control apolipoproteins when presented to cells on the surface of spherical r-HDL particles. These results provided the first evidence that the apolipoprotein fraction of tyrosylated HDL is responsible for its increased ability to stimulate cellular cholesterol efflux. The fact that the difference in the abilities of control and tyrosylated apolipoproteins to stimulate cholesterol efflux was seen only when they were incorporated into spherical r-HDL, but not discoidal r-HDL, suggests that the conformation of the crosslinked apolipoproteins plays an important role in their ability to stimulate efflux (see below).

The results of experiments incorporating tyrosyl radical-oxidized mixtures of apo A-I and apo A-II into spherical r-HDL suggest that an AI-AII crosslinked species is involved in the enhancement of cellular cholesterol efflux by tyrosylated HDL. Furthermore, tyrosyl radical-mediated oxidation of HDL containing both apo A-I and apo A-II (LpAI/AII), but not HDL containing only apo A-I (LpAI) or apo A-II (AII-only HDL), produced particles with an enhanced ability to stimulate cholesterol efflux. When protein fractions enriched in specific crosslinked apolipoprotein species of tyrosylated HDL were presented to cells

on the surface of spherical r-HDL, the AI-$(AII)_2$ species enhanced cholesterol efflux to the greatest extent.

Purification of crosslinked apolipoprotein species using reverse phase HPLC, anion exchange FPLC, and size exclusion HPLC proved to be more difficult than was initially expected. Apo A-I and apo A-II are both exchangeable apolipoproteins consisting primarily of amphipathic alpha-helices. We did not know how the properties of the proteins would be affected by tyrosyl radical-mediated oxidation, although previous studies had demonstrated that the overall charge of tyrosylated HDL was unchanged (Francis *et al.* 1993). Since the crosslinked apolipoprotein species were not separated by reversed phase or anion exchange chromatography, they must be similar in hydrophobicity and charge. Optimization of these methods might have resulted in enhanced separation of the apolipoprotein species; however, we instead chose preparative electrophoresis as a means of quickly and directly purifying the species. As the protein species were not separated by non-denaturing gradient gel electrophoresis, this necessitated using SDS; the SDS was successfully removed by AG-11 A8 column chromatography.

There has been some debate regarding whether a high plasma HDL level directly protects against atherosclerosis by inducing reverse cholesterol transport, or whether other properties of HDL might be responsible. As was discussed in Chapter 1, several studies in animal models suggest that HDL is directly protective, and reverse cholesterol transport is generally accepted as its primary atheroprotective mechanism. Our results

suggest that oxidation of HDL by tyrosyl radical enhances cholesterol efflux, the rate limiting step of reverse cholesterol transport. Since the LpAI/AII subfraction comprises 49 to 75% of HDL in plasma from normal subjects (Cheung & Albers 1984), we expect that formation of such apolipoprotein AI-AII crosslinked species may be a significant means of enhancing the atheroprotective effects of HDL *in vivo*.

As was mentioned above, the conformation of the crosslinked apolipoproteins on the surface of reconstituted HDL particles appears to affect their ability to enhance cellular cholesterol efflux. This observation correlates with previous studies using reconstituted particles, which have shown that the conformation of apo A-I depends on the particle size and shape and upon the presence of other apolipoproteins. On discoidal r-HDL, apo A-I binds along the edges of the disc, interacting with phospholipid acyl chains (Bergeron *et al.* 1995; Calabresi *et al.* 1993; Durbin & Jonas 1997). The conformation of the central hinged domain (residues 99-143) of apo A-I varies with the diameter of the particle (Calabresi *et al.* 1993); specifically, as the particle size increases, an α-helical portion of the hinge (residues 99-121) increasingly interacts with lipids, causing structural rearrangement of apo A-I (Bergeron *et al.* 1995). Furthermore, the conformation of apo A-I on spherical r-HDL (93 Å) is similar to that on large discoidal r-HDL (96 Å), but distinct from that on smaller discoidal particles (78 Å) (Jonas *et al.* 1990), which suggests that the central hinge is lipid-bound on spherical r-HDL as well. Binding of apo A-II to discoidal r-HDL (98 Å) alters the conformation of apo A-I by displacing its central four α-helices (residues 99-187) from contact with the lipid surface (Durbin & Jonas 1997).

The conformation of the apolipoproteins may be further altered by introduction of crosslinks during tyrosyl radical-mediated oxidation. The nature of the crosslink is unknown at present. It could be formed by reaction of a lipid peroxidation product with the proteins, by reaction of two oxidized tyrosine residues to form a dityrosine cross-bridge, or by reaction of tyrosyl radical with a lipid component of HDL. However, other methods of causing lipoprotein peroxidation produce similar crosslinking patterns in HDL apolipoproteins (Marcel *et al.* 1989), but do not produce oxidized HDL that enhance cellular cholesterol efflux (Bonnefort-Rousselot *et al.* 1995; Morel 1994; Nagano *et al.* 1991; Rifici & Khachadurian 1996a; Rifici & Khachadurian 1996b). Therefore it is likely that the crosslink formed is unique to tyrosyl radical-mediated oxidation. If it were a protein-bound dityrosine crosslink, it should be possible to predict the location of the crosslink and the resulting apolipoprotein conformation based on the amino acid sequences and predicted secondary structures of apo A-I and apo A-II, the number of apolipoproteins on the surface of HDL, and the present knowledge of their tertiary structure when bound to the surface of HDL. Future studies will determine the site of crosslinking by peptide sequencing. Site directed mutagenesis of recombinant apo A-I and apo A-II could then be used to confirm the importance of specific amino acid residues in the generation of crosslinked protein species capable of enhancing cholesterol efflux.

The apo A-I(A-II)$_2$ crosslinked species showed increased ability to stimulate cellular cholesterol efflux, while the apo A-I/A-II crosslinked species showed little change in its

activity (Figure 6.6). *In vivo*, apo A-II is found as a homodimer linked by a disulfide bridge at Cys-6, not as a monomer (Brewer *et al.* 1986). Perhaps dimerization of apo A-II is necessary for the crosslinked species to assume its active conformation. This hypothesis is supported by changes in the secondary structure of apo A-II associated with reduction of the disulfide bridge: monomeric apo A-II shows a decrease in its α-helical content (Lund-Katz *et al.* 1996). In contrast to our observations of crosslink activity, reduction of the disulfide bridge to produce HDL or r-HDL containing only monomeric apo A-II has been shown to increase the ability of the particles to remove cholesterol from the plasma membrane and intracellular stores (Bernini *et al.* 1996; Lund-Katz *et al.* 1996). Perhaps this methodology could be used to further test whether the apo A-I$(A\text{-}II)_2$ crosslinked species is required for enhanced stimulation of cellular cholesterol efflux by tyrosylated HDL.

Apo A-I^{Milano} is a mutant form of apo A-I in which cysteine is substituted for arginine at amino acid 173, forming a disulfide linked homodimer. Individuals with apo A-I^{Milano} have decreased plasma HDL and apo A-I levels, and show decreased incidence of atherosclerotic disease (Franceschini *et al.* 1980). The decreased levels of HDL and apo A-I and the atheroprotective effects of apo A-I^{Milano} are felt to be caused by accelerated turnover of HDL containing the mutant protein (Roma *et al.* 1993). It has also been shown that apo A-I^{Milano} dimers have a marked difference in conformation compared to native apo A-I both in solution and on reconstituted HDL particles (Calabresi *et al.* 1997; Calabresi *et al.* 1994), with the conformation of lipid-free apo A-I^{Milano} being similar to that of lipid-bound apo A-I . It is possible that the atheroprotective effect of apo A-I^{Milano}

depends in part on changes in the conformation of the apolipoprotein A-I(A-II)$_2$ crosslinks further increasing their ability to stimulate cholesterol efflux; it might also be that the altered conformation of apo A-I^{Milano} facilitates production of apolipoprotein A-I(A-II)$_2$ crosslinks. To test these hypotheses, recombinant apo A-I^{Milano} could be used in place of native apo A-I in experiments similar to those described in section 4.2.4.

Previous work in our laboratory (Francis *et al.* 1993; Francis *et al.* 1996) has shown that tyrosyl radical-oxidized HDL stimulates cellular cholesterol efflux by diverting intracellular cholesterol from an ACAT-accessible pool to an efflux-accessible domain on the cell surface. Many questions remain to be answered about the mechanism by which this occurs. It is not known if tyrosylated HDL stimulates translocation of intracellular cholesterol by the same pathway as HDL, or a novel pathway. It is also unclear if Tyr-HDL binds at a cell-surface receptor, or if it produces a signal by perturbation of the plasma membrane. Whether it increases cholesterol transport from an intracellular site by caveolae, transport vesicles, or a cholesterol transport protein is also unknown. Perhaps Tyr-HDL blocks transport of plasma membrane cholesterol to the endoplasmic reticulum for esterification by ACAT, as progesterone does (Mazzone *et al.* 1995; Metherall *et al.* 1996), and thus increases the size of the efflux-accessible pool.

We envision a pathway for cholesterol efflux from cells in which HDL or lipid-free apolipoproteins bind to a cell surface receptor or plasma membrane domain, producing a signal which activates protein kinase C (Li & Yokoyama 1995; Mendez *et al.* 1991; Oram & Yokoyama 1996; Theret *et al.* 1990), eventually leading to translocation of

cholesterol from the ACAT-accessible pool to the efflux-accessible pool. Tyrosyl radical-mediated oxidation of HDL leads to formation of apo AI-AII crosslinks, which may alter the conformation of the apolipoproteins. The crosslinked apolipoproteins might be stimulating cholesterol efflux by the same pathway as control HDL apolipoproteins, or by an independent mechanism. Since apo A-II has a higher lipid affinity than apo A-I, crosslinking may tether apo A-I to the surface of HDL and prevent its complete dissociation from the particle. If it prevents dissociation of apo A-I, this may result in prolonged stimulation of the apolipoprotein-mediated pathway. Alternately, the crosslinked apolipoproteins may stimulate a novel pathway for cholesterol efflux.

Future studies in our laboratory will focus on purification to homogeneity of crosslinked apolipoprotein species from tyrosyl radical-oxidized HDL for use in reconstitution experiments. We will aim to confirm whether AI-AII crosslinked species are responsible for enhancement of cholesterol efflux by tyrosylated HDL, and whether short crosslinked peptide fragments could also be used to stimulate cholesterol efflux. This will provide valuable tools for the study of the critical first step of reverse cholesterol transport, and allow elucidation of the pathway(s) for intracellular cholesterol transport and cholesterol efflux. Further understanding of these mechanisms may suggest potential therapies for the treatment and prevention of atherosclerosis.

Bibliography

Acton, S., Rigotti, A., Landschulz, K. T., Xu, S., Hobbs, H. H., Krieger, M. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271:518-520

Acton, S. L., Scherer, P. E., Lodish, H. F., Krieger, M. 1994. Expression cloning of SR-BI, a CD36-related Class B Scavenger Receptor. *J. Biol. Chem.* 269:21003-21009

Amado, R., Aeschbach, R., Neukom, H. 1984. Dityrosine: in vitro production and characterization. *Methods Enzymol.* 107:377-388

Andersson, L.-O. 1997. Pharmacology of apolipoprotein A-I. *Cur. Opinion Lipidol.* 8:225-228

Assman, G., Eckardstein, A. v., Funke, H. 1993. High density lipoproteins, reverse transport of cholesterol, and coronary artery disease: insights from mutations. *Circulation* 87, Suppl. III:28-34

Badimon, J. J., Badimon, L., Fuster, V. 1990. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. *J. Clin. Invest.* 85:1234-1241

Barbaras, R., Puchois, P., Fruchart, J.-C., Ailhaud, G. 1987. Cholesterol efflux from cultured adipose cells is mediated by LpAI particles but not by LpAI:AII particles. *Biochem. Biophys. Res. Commun.* 142:63-69

Barkia, A., Puchois, P., Ghalim, N., Torpier, G., Barbaras, R., *et al.* 1991. Differential role of apolipoprotein AI-containing particles in cholesterol efflux from adipose cells. *Atheroscler.* 87:135-146

Barter, P. J., Rye, K.-A. 1996a. High density lipoproteins and coronary heart disease. *Atheroscler.* 121:1-12

Barter, P. J., Rye, K.-A. 1996b. Molecular mechanisms of reverse cholesterol transport. *Cur. Opinion Lipidol.* 7:82-87

Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466-468

Bergeron, J., Frank, P. G., Scales, D., Meng, Q.-H., Castro, G., Marcel, Y. L. 1995. Apolipoprotein A-I conformation in reconstituted discoidal lipoproteins varying in phospholipid and cholesterol content. *J. Biol. Chem.* 270:27429-27438

Bergmark, C., Wu, R., deFaire, U., Lefvert, A. K., Swedenborg, J. 1995. Patients with early-onset peripheral vascular disease have increased levels of autoantibodies against oxidized LDL. *Arterioscler. Thromb. Vasc. Biol.* 15:441-445

Berliner, J. A., Heinecke, J. W. 1996. The role of oxidized lipoproteins in atherogenesis. *Free Radic. Biol. Med.* 20:707-727

Bernini, F., Calabresi, L., Bonfadini, G., Francescini, G. 1996. The molecular structure of apolipoprotein A-II modulates the capacity of HDL to promote cell cholesterol efflux. *Biochim. Biophys. Acta* 1299:103-109

Bielicki, J. K., Johnson, W. J., Weinberg, R. B., Glick, J. M., Rothblat, G. H. 1992. Efflux of lipid from fibroblasts to apolipoproteins: dependence on elevated levels of cellular unesterified cholesterol. *J. Lipid Res.* 33:1699-1709

Blum, C. B., Dell, R. B., Palmer, R. H., Ramakrishnan, R., Seplowitz, A. H., Goodman, D. S. 1985. Relationship of the parameters of body cholesterol metabolism with

plasma levels of HDL cholesterol and the major HDL apoproteins. *J. Lipid Res.* 26:1079-1088

Bond, H. M., Morrone, G., Venuta, S., Howell, K. E. 1991. Characterization and purification of proteins which bind high-density lipoprotein: a putative cell-surface receptor. *Biochem. J.* 279:633-641

Bonnefort-Rousselot, D., Motta, C., Khalil, A. O., Sola, R., LaVille, A. E., *et al.* 1995. Physicochemical changes in human high-density lipoproteins (HDL) oxidized by gamma radiolysis-generated oxyradicals. Effect on their cholesterol effluxing capacity. *Biochim. Biophys. Acta* 1255:23-30

Borensztajn, J., Getz, G. S., Padley, R. J., Kotlar, T. J. 1982. The apoprotein B-independent hepatic uptake of chylomicron remnants. *Biochem. J.* 204:609-612

Borensztajn, J., Kotlar, T. J. 1984. Liver uptake of chylomicron remnants with high and low apoprotein E:C ratios. *Proc. Natl. Acad. Sci. USA* 81:5863-5866

Borensztajn, J., Kotlar, T. J., Chang, S. 1991. Apoprotein-independent binding of chylomicron remnants to rat liver membranes. *Biochem. J.* 279:769-773

Bostrom, K., Watson, K. E., Stanford, W. P., Demer, L. L. 1995. Atherosclerotic calcification: relation to developmental osteogenesis. *Am. J. Cardiol.* 75:88B-91B

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254

Breslow, J. L. 1996. Mouse models of atherosclerosis. *Science* 272:685-688

Brewer, H. B., Ronan, R., Meng, M., Bishop, C. 1986. Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. *Methods Enzymol.* 128:223-246

Brinton, E. A., Oram, J. F., Chen, C.-H., Albers, J. J., Bierman, E. L. 1986. Binding of high density lipoprotein to cultured fibroblasts after chemical alteration of apoprotein amino acid residues. *J. Biol. Chem.* 261:495-503

Brown, M. S., Ho, Y. K., Goldstein, J. L. 1980. The cholesteryl ester cycle in macrophage foam cells. *J. Biol. Chem.* 255:9344-9352

Burcham, P. C., Kuhan, Y. T. 1997. Diminished susceptibility to proteolysis after protein modification by the lipid peroxidation product malondialdehyde: inhibitory role for crosslinked and noncrosslinked adducted proteins. *Arch. Biochem. Biophys.* 340:331-337

Calabresi, L., Franceschini, G., Burkybile, A., Jonas, A. 1997. Activation of lecithin cholesterol acyltransferase by a disulfide-linked apolipoprotein A-I dimer. *Biochemical and Biophysical Research Communication* 232:346-349

Calabresi, L., Meng, Q.-H., Castro, G. R., Marcel, Y. L. 1993. Apolipoprotein A-I conformation in discoidal particles: Evidence for alternate structures. *Biochemistry* 32:6477-6484

Calabresi, L., Vecchio, G., Longhi, R., Gianazza, E., Palm, G., *et al.* 1994. Molecular characterization of native and recombinant apolipoprotein A-IMilano dimer. *J. Biol. Chem.* 269:32168-32174

Castro, G., Nihoul, L. P., Dengremont, C., deGeitere, C., Delfly, B., *et al.* 1997. Cholesterol efflux, lecithin-cholesterol acyltransferase activity, and pre-beta particle formation by serum from human apolipoprotein A-I and apolipoprotein A-I/apolipoprotein A-II transgenic mice consistent with the latter being less effective for reverse cholesterol transport. *Biochemistry* 36:2243-2249

Castro, G. R. 1997. Role of apolipoproteins A-I and A-II in reverse cholesterol transport. In *Satelite Symposium on Reverse Cholesterol Transport, 11th International Symposium on Atheroscler.* Lille, France:

Cheung, M. C., Albers, J. J. 1984. Characterization of lipoprotein particles isolated by immunoaffinity chromatography. *J. Biol. Chem.* 259:12201-12209

Chung, B. H., Wilkinson, T., Greer, J. C., Segrest, J. P. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* 21:284-291

Coffey, M. D., Cole, R. A., Colles, S. M., Chisolm, G. M. 1995. In vitro cell injury by oxidized low density lipoprotein involves lipid hydroperoxide-induced formation of alkoxyl, lipid, and peroxyl radicals. *J. Clin. Invest.* 96:1866-1873

Cohen, R. D., Castellani, L. W., Qiao, J. H., vanLenten, B. J., Lusis, A. J., Reue, K. 1997. Reduced aortic lesions and elevated high density lipoprotein levels in transgenic mice overexpressing mouse apolipoprotein A-IV. *J. Clin. Invest.* 99:1906-1916

Colles, S. M., Irwin, K. C., Chisolm, G. M. 1996. Roles of multiple oxidized LDL lipids in cellular injury: dominance of 7-beta-hydroperoxycholesterol. *J. Lipid Res.* 37:2018-2028

Daugherty, A., Dunn, J. L., Rateri, D. L., Heinecke, J. W. 1994. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest.* 94:437-444

Davidson, W. S., Lund-Katz, S., Johnson, W. J., Anantharamaiah, G. M., Palgunachari, M. N., *et al.* 1994. The influence of apolipoprotein structure on the efflux of

cellular free cholesterol to high density lipoprotein. *J. Biol. Chem.* 269:22975-22982

Davidson, W. S., Rodrigueza, W. V., Lund-Katz, S., Johnson, W. J., Rothblat, G. H., Phillips, M. C. 1995. Effects of acceptor particle size on the efflux of cellular free cholesterol. *J. Biol. Chem.* 270:17106-17113

Decossin, C., Tailleux, A., Fruchart, J.-C., Fievet, C. 1995. Prevention of in vitro low-density lipoprotein oxidation by an albumin-containing Lp A-I subfraction. *Biochim. Biophys. Acta* 1255:31-38

Dietschy, J. M. 1997. Theoretical considerations of what regulates low-density-lipoprotein and high-density-lipoprotein cholesterol. *Am. J. Clin. Nutr. Suppl.* 65:1581s-1589s

Dinchuk, J., Hart, J., Gonzalez, G., Karmann, G., Schmidt, D., Wirak, D. O. 1995. Remodelling of lipoproteins in transgenic mice expressing human cholesteryl ester transfer protein. *Biochim. Biophys. Acta* 1255:301-310

Durbin, D. M., Jonas, A. 1997. The effect of apolipoprotein A-II on the structure and function of apolipoprotein A-I in a homogeneous reconstituted high density lipoprotein particle. *J. Biol. Chem.* 272:31333-31339

Duverger, N., Ghalim, N., Theret, N., Fruchart, J. C., Castro, G. 1994. Lipoproteins containing apolipoprotein A-IV: Composition and relation to cholesterol esterification. *Biochim. Biophys. Acta* 1211:23-28

Edelstein, C., Halari, M., Scanu, A. M. 1982. On the mechanism of the displacement of apolipoprotein A-I by apolipoprotein A-II from the high density lipoprotein surface. *J. Biol. Chem.* 257:7189-7195

Fidge, N. H., Nestel, P. J. 1985. Identification of apolipoproteins involved in the interaction of human high density lipoprotein 3 with receptors on cultured cells. *J. Biol. Chem.* 260:3570-3575

Fielding, C. J., Fielding, P. E. 1995a. Molecular physiology of reverse cholesterol transport. *J. Lipid Res.* 36:211-228

Fielding, P. E., Fielding, C. J. 1995b. Plasma membrane caveolae mediate the efflux of cellular free cholesterol. *Biochemistry* 34:14288-14292

Fielding, P. E., Fielding, C. J. 1996. Intracellular transport of low density lipoprotein derived free cholesterol begins at clathrin-coated pits and terminates at cell surface caveolae. *Biochemistry* 35:14932-14938

Franceschini, G., Sirtori, C. R., Capurso, A., Weisgraber, K. H., Mahley, R. W. 1980. A-I Milano apoprotein: Decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *J. Clin. Invest.* 66:892-900

Francis, G. A., Knopp, R. H., Oram, J. F. 1995. Defective removal of cellular cholesterol and phospholipids by apolipoprotein A-I in Tangier disease. *J. Clin. Invest.* 96:78-87

Francis, G. A., Mendez, A. J., Bierman, E. L., Heinecke, J. W. 1993. Oxidative tyrosylation of high density lipoprotein by peroxidase enhances cholesterol removal from cultured fibroblasts and macrophage foam cells. *Proc. Natl. Acad. Sci. USA* 90:6631-6635

Francis, G. A., Oram, J. F., Heinecke, J. W., Bierman, E. L. 1996. Oxidative tyrosylation of HDL enhances the depletion of cellular cholesteryl esters by a mechanism independent of passive sterol desorption. *Biochemistry* 35:15188-15197

Francis, G. A., Roberts, C., Perry, R. J. 1997. Activated human phagocytes modify high density lipoprotein to a form that enhances cellular cholesterol efflux. *Atheroscler.* 134:366-367

Gillotte, K. L., Davidson, W. S., Lund-Katz, S., Rothblat, G. H., Phillips, M. C. 1996. Apolipoprotein A-I structural modification and the functionality of reconstituted high density lipoprotein particles in cellular cholesterol efflux. *J. Biol. Chem.* 271:23792-23798

Glomset, J. A. 1968. The plasma lecithin:cholesterol acyltransferase reaction. *J. Lipid Res.* 9:155-167

Goldstein, J. L., Ho, Y. K., Basu, S. K., Brown, M. S. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA* 76:333-337

Gordon, T., Castelli, W. P., Hjortland, M. C., Kannel, W. B., Dawber, T. R. 1977. High Density Lipoprotein As a Protective Factor Against Coronary Heart Disease: The Framingham Study. *Am. J. Med.* 62:707-714

Graham, D. L., Oram, J. F. 1987. Identification and characterization of a high density lipoprotein binding protein in cell membrane by ligand blotting. *J. Biol. Chem.* 262:7439-7442

Guyton. 1994. The arterial wall and the atherosclerotic lesion. *Cur. Opinion Lipidol.* 5:376-381

Guyton, J. R., Klemp, K. F. 1996. Development of the lipid-rich core in human atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 16:4-11

Hammer, A., Kager, G., Dohr, G., Rabl, H., Ghassempur, I., Jurgens, G. 1995. Generation, characterization, and histochemical application of monoclonal antibodies selectively recognizing oxidatively modified apoB-containing serum lipoproteins. *Arterioscler. Thromb. Vasc. Biol.* 15:704-713

Hara, H., Yokoyama, S. 1991. Interaction of free apolipoproteins with macrophages. *J. Biol. Chem.* 266:3080-3086

Hayek, T., Masucci-Magoulas, L., Jiang, X., Walsh, A., Rubin, E., *et al.* 1995. Decreased early atherosclerotic lesions in hypertriglyceridemic mice expressing cholesteryl ester transfer protein transgene. *J. Clin. Invest.* 96:2071-2074

Heinecke, J. W., Li, W., Daehnke, H. L., Goldstein, J. A. 1993a. Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages. *J. Biol. Chem.* 268:4069-4077

Heinecke, J. W., Li, W., Francis, G. A., Goldstein, J. A. 1993b. Tyrosyl radical generated by myeloperoxidase catalyses the oxidative cross-linking of proteins. *J. Clin. Invest.* 91:2866-2872

Hokland, B. M., Mendez, A. J., Oram, J. F. 1992. Cellular localization and characterization of proteins that bind high density lipoprotein. *J. Lipid Res.* 33:1335-1342

Jacob, J. S., Cistola, D. P., Hsu, F. F., Muzaffar, S., Mueller, D. M., *et al.* 1996. Human phagocytes employ the myeloperoxidase-hydrogen peroxide system to synthesize

dityrosine, trityrosine, pulcherosine, and isodityrosine by a tyrosyl radical-dependent pathway. *J. Biol. Chem.* 271:19950-19956

Ji, Y., Jian, B., Wang, N., Sun, Y., Moya, M. d. l. L., *et al.* 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* 272:20982-20985

Jialal, I., Devaraj, S. 1996. The role of oxidized low density lipoprotein in atherogenesis. *J. Nutr.* 126:1053s-1057s

Johnson, W. J., Kilsdonk, E. P. C., Tol, A. v., Phillips, M. C., Rothblat, G. H. 1991a. Cholesterol efflux from cells to immunopurified subfractions of human high density lipoprotein: LP-AI and LP-AI/AII. *J. Lipid Res.* 32:1993-2000

Johnson, W. J., Mahlberg, F. H., Rothblat, G. H., Phillips, M. C. 1991b. Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta* 1085:273-298

Jonas, A. 1986. Reconstitution of High-Density Lipoproteins. *Methods Enzymol.* 128:553-582

Jonas, A., Wald, J. H., Toohill, K. L. H., Krul, E. S., Kezdy, K. E. 1990. Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J. Biol. Chem.* 265:22123-22129

Jurasek, L., Olafson, R. W., Johnson, P., Smillie, L. B. 1976. Relationships between the structures and activities of some microbial serine proteases.

I. Purification, enzymic properties and primary structures of Streptomyces griseus proteases A, B, and trypsin. In *Proteolysis and Physiological Regulation*, ed. D. Ribbons, K. Brew. pp. 93-123. New York: Academic Press

Kannel, W. B., Gordon, T., Castelli, W. P. 1981. Role of lipids and lipoprotein fractions in atherogenesis: The Framingham study. *Prog. Lipid Res.* 20:339-348

Kapp, O. H., Vinogradov, S. N. 1978. Removal of sodium dodecyl sulfate from proteins. *Anal. Biochem.* 91:230-235

Kates, M. 1986. *Techniques of Lipidology: Isolation, analysis, and identification of lipids*. Second revised ed. Vol. 3, part 2. New York: Elsevier

Klebanoff, S. J. 1980. Oxygen metabolism and the toxic properties of phagocytes. *Ann. Intern. Med.* 93:480-489

Klimov, A. N., Gurevich, V. S., Nikiforova, A. A., Shatilina, L. V., Kuzmin, A. A., *et al.* 1993. Antioxidative activity of high density lipoproteins in vivo. *Atheroscler.* 100:13-18

Kouzuma, R., Tasaki, H., Komura, T., Nakashima, Y., Kuroiwa, A., *et al.* 1995. Combined treatment of probucol with diltiazem regresses atherosclerosis induced by 1% cholesterol diet in rabbit aorta. *Artery* 21:337-351

Kozarsky, K. F., Donahee, M. H., Rigotti, A., Iqbal, S. N., Edelman, E. R., Krieger, M. 1997. Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature* 387:414-417

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685

Lagocki, P. A., Scanu, A. M. 1980. In vitro modulation of the apolipoprotein composition of high density lipoprotein. *J. Biol. Chem.* 255:3701-3706

Lagrost, L., Dengremont, C., Athias, A., Geitere, C. d., Fruchart, J.-C., *et al.* 1995. Modulation of cholesterol efflux from Fu5AH hepatoma cells by the

apolipoprotein content of high density lipoprotein particles. *J. Biol. Chem.* 270:13004-13009

Lagrost, L., Persegol, L., Lallemant, C., Gambert, P. 1994. Influence of apolipoprotein composition of high density lipoprotein particles on cholesteryl ester transfer protein activity. *J. Biol. Chem.* 269:3189-3197

Leeuwenburgh, C., Rasmussen, J. E., Hsu, F. F., Mueller, D. M., Pennathur, S., Heinecke, J. W. 1997. Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques. *J. Biol. Chem.* 272:3520-3526

Li, H., Reddick, R. L., Maeda, N. 1993. Lack of apoA-I is not associated with increased susceptibility to atherosclerosis in mice. *Arterioscler. Thromb.* 13:1814-1821

Li, Q., Yokoyama, S. 1995. Independent regulation of cholesterol incorporation into free apolipoprotein-mediated cellular lipid efflux in rat vascular smooth muscle cells. *J. Biol. Chem.* 270:26216-26223

Liu, H., Malhotra, V., Ryan, R. O. 1991. Displacement of apolipophorin III from the surface of low density lipoprotein by human apolipoprotein A-I. *Biochem. Biophys. Res. Commun.* 179:734-740

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275

Lund-Katz, S., Murley, Y. M., Yon, E., Gillotte, K. L., Davidson, W. S. 1996. Comparison of the structural and functional effects of monomeric and dimeric human apolipoprotein A-II in high density lipoprotein particles. *Lipids* 31:1107-1113

Mackness, M. I., Arrol, S., Durrington, P. N. 1991. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.* 286:152-154

Maier, J. A. M., Barenghi, L., Pagani, F., Bradamante, S., Comi, P., Ragnotti, G. 1994. The protective role of high-density lipoprotein on oxidized-low-density-lipoprotein-induced U937/endothelial cell interactions. *Eur. J. Biochem.* 221:35-41

Mamo, J. C. L., Yu, K. C. W., Elsegood, C. L., Smith, D., Vine, D., *et al.* 1997. Is atherosclerosis exclusively a postprandial phenomenon? *Clin. Exp. Pharmacol. Physiol.* 24:288-293

Marcel, Y. L., Jewer, D., Leblond, L., Weech, P. K., Milne, R. R. 1989. Lipid peroxidation changes the expression of specific epitopes of apolipoprotein A-I. *J. Biol. Chem.* 264:19942-19950

Matsumoto, A., Mitchell, A., Kurata, H., Pyle, L., Kondo, K., *et al.* 1997. Cloning and characterization of HB2, a candidate high density lipoprotein receptor. *J. Biol. Chem.* 272:16778-16782

Matz, C. E., Jonas, A. 1982. Micellar complexes of human apolipoprotein A-I with phosphatidylcholines and cholesterol prepared from cholate-lipid dispersions. *J. Biol. Chem.* 257:4535-4540

Mazzone, T., Krishna, M., Lange, Y. 1995. Progesterone blocks intracellular translocation of free cholesterol derived from cholesteryl ester in macrophages. *J. Lipid Res.* 36:544-551

McKnight, G. L., Reasoner, J., Gilbert, T., Sundquist, K. O., Hokland, B., *et al.* 1992. Cloning and expression of a cellular high density lipoprotein-binding protein that is up-regulated by cholesterol loading of cells. *J. Biol. Chem.* 267:12131-12141

Mendez, A. J. 1997. Cholesterol efflux mediated by apolipoproteins is an active cellular process distinct from efflux mediated by passive diffusion. *J. Lipid Res.* 38:1807-1821

Mendez, A. J., Anantharamaiah, G. M., Segrest, J. P., Oram, J. F. 1994a. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J. Clin. Invest.* 94:1698-1705

Mendez, A. J., Anantharamaiah, G. M., Segrest, J. P., Oram, J. F. 1994b. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J. Clin. Invest.* 94:1698-1705

Mendez, A. J., Lin, G., Oram, J. F. 1997. Apolipoprotein-mediated lipid efflux does not derive from membrane caveolae domains. *Circulation* 96:I-485

Mendez, A. J., Oram, J. F. 1997. Limited proteolysis of high density lipoprotein abolishes its interaction with cell-surface binding sites that promote cholesterol efflux. *Biochim. Biophys. Acta* 1346:285-299

Mendez, A. J., Oram, J. F., Bierman, E. L. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* 266:10104-10111

Mendez, A. J., Uint, L. 1996. Apolipoprotein-mediated cellular cholesterol and phospholipid efflux depend on a functional Golgi apparatus. *J. Lipid Res.* 37:2510-2524

Metherall, J. E., Waugh, K., Li, H. 1996. Progesterone inhibits cholesterol biosynthesis in cultured cells. *J. Biol. Chem.* 271:2627-2633

Miller, N. E., Thelle, D. S., Forde, O. H., Mjos, O. D. 1977. The Tromso Heart Study: High Density Lipoprotein and Coronary Heart Disease: A Prospective Case-Control Study. *Lancet* 1:965-968

Mironova, M., Virella, G., Lopes-Virella, M. F. 1996. Isolation and characterization of human antioxidized LDL autoantibodies. *Arterioscler. Thromb. Vasc. Biol.* 16:222-229

Mitchell, G. A., Lambert, M., Tanguay, R. M. 1995. Hypertyrosinemia. In *The Metabolic and Molecular Bases of Inherited Disease*, ed. C. R. Scriver, A. L. Beaudet, W. S. Sly, D. Valle. pp. 1077-1106. Vol. 1

Morel, D. W. 1994. Reduced cholesterol efflux to mildly oxidized high density lipoprotein. *Biochem. Biophys. Res. Commun.* 200:408-416

Mowri, H.-O., Patsch, W., Smith, L. C., Antonio M Gotto, J., Patsch, J. R. 1992. Different reactivities of high density lipoprotein 2 subfractions with hepatic lipase. *J. Lipid Res.* 33:1269-1279

Nagano, Y., Arai, H., Kita, T. 1991. High density lipoprotein loses its effect to stimulate efflux of cholesterol from foam cells after oxidative modification. *Proc. Natl. Acad. Sci. USA* 88:6457-6461

Navab, M., Berliner, J. A., Watson, A. D., Hama, S. Y., Territo, M. C., *et al.* 1996. The yin and yang of oxidation in the development of the fatty streak - a review based on the 1994 George Lyman Duff Memorial Lecture. *Arterioscler. Thromb. Vasc. Biol.* 16:831-842

Nichols, A. V., Blanche, P. J., Shore, V. G., Gong, E. L. 1989. Conversion of apolipoprotein-specific high-density lipoprotein populations during incubation of human plasma. *Biochim. Biophys. Acta* 1001:325-337

North, M. J. 1989. Prevention of unwanted proteolysis. In *Proteolytic enzymes: a practical approach*, ed. R. J. Beynon, J. S. Bond. pp. 105-123. Oxford: IRL Press

Oikawa, S., Mendez, A. J., Oram, J. F., Bierman, E. L., Cheung, M. C. 1993. Effects of high-density lipoprotein particles containing apo A-I, with or without apo A-II, on intracellular cholesterol efflux. *Biochim. Biophys. Acta* 1165:327-334

Olsson, A. G., Yuan, X. M. 1996. Antioxidants in the prevention of atherosclerosis. *Cur. Opinion Lipidol.* 7:374-380

Oram, J. F., Brinton, E. A., Bierman, E. L. 1983. Regulation of HDL receptor activity in cultured human skin fibroblasts and human arterial smooth muscle cells. *J. Clin. Invest.* 72:1611-1621

Oram, J. F., Mendez, A. J., Slotte, J. P., Johnson, T. F. 1991. High density lipoprotein apolipoproteins mediate removal of sterol from intracellular pools but not from plasma membranes of cholesterol-loaded fibroblats. *Arterioscler. Thromb.* 11:403-414

Oram, J. F., Yokoyama, S. 1996. Apolipoprotein-mediated removal of cellular cholesterol and phospholipids. *J. Lipid Res.* 37:2473-2491

Osono, Y., Woollett, L. A., Marotti, K. R., Melchior, G. W., Dietschy, J. M. 1996. Centripetal cholesterol flux from extrahepatic organs to the liver is independent of the concentration of high density lipoprotein-cholesterol in plasma. *Proc. Natl. Acad. Sci. USA* 93:4114-4119

Palinski, W., Horkko, S., Miller, E., Steinbrecher, U. P., Powell, H. C., *et al.* 1996. Cloning of monoclonal autoantibodies to epitopes of oxidized lipoproteins from apolipoprotein E-deficient mice. *J. Clin. Invest.* 98:800-814

Palinski, W., Ord, V. A., Plump, A. S., Breslow, J. L., Steinberg, D., Witztum, J. L. 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis. *Arterioscler. Thromb.* 14:605-616

Palinski, W., Rosenfeld, M. E., Yla-Herttuala, S., Gurtner, G. C., Socher, S. S., *et al.* 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA* 86:1372-1376

Palinski, W., Tangirala, R. K., Miller, E., Young, S. G., Witztum, J. L. 1995. Increased autoantibody titers against epitopes of oxidized LDL in LDL receptor-deficient mice with increased atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 15:1569-1576

Parthasarathy, S., Barnett, J., Fong, L. G. 1990. High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim. Biophys. Acta* 1044:275-283

Paszty, C., Maeda, N., Verstuyft, J., Rubin, E. M. 1994. Apolipoprotein AI transgene corrects apolipoprotein E deficiency-induced atherosclerosis in mice. *J. Clin. Invest.* 94:899-903

Phillip, M., Bender, M. L. 1983. Kinetics of subtilisin and thiolsubtilisin. *Mol. Cell. Biochem.* 51:5-32

Pittman, R. C., Glass, C. C., Atkinson, D., Small, D. M. 1987. Synthetic high density lipoprotein particles. *J. Biol. Chem.* 262:2435-2442

Plump, A. S., Azrolan, N., Odaka, H., Wu, L., Jiang, X., *et al.* 1997. ApoA-I kockout mice: characterization of HDL metabolism in homozygotes and identification of a post-RNA mechanism of apoA-I up-regulation in heterozygotes. *J. Lipid Res.* 38:1033-1047

Plump, A. S., Scott, C. J., Breslow, J. L. 1994. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc. Natl. Acad. Sci. USA* 91:9607-9611

Quintao, E. C. R. 1995. Is reverse cholesterol transport a misnomer for suggesting its role in the prevention of atheroma formation? *Atheroscler.* 116:1-14

Rabilloud, T., Carpentier, G., Tarroux, P. 1988. Improvement and simpification of low-background silver staining of proteins by using sodium dithionite. *Electrophoresis* 9:288-291

Rifici, V. A., Khachadurian, A. K. 1996a. Effects of dietary vitamin C and E supplementation on the copper mediated oxidation of HDL and on HDL mediated cholesterol efflux. *Atheroscler.* 127:19-26

Rifici, V. A., Khachadurian, A. K. 1996b. Oxidation of high density lipoproteins: characterization and effects on cholesterol efflux from J774 macrophages. *Biochim. Biophys. Acta* 1299:87-94

Rigotti, A., Trigatti, B., Babitt, J., Penman, M., Xu, S., Krieger, M. 1997a. Scavenger receptor BI - a cell surface receptor for high density lipoprotein. *Cur. Opinion Lipidol.* 8:181-188

Rigotti, A., Trigatti, B. L., Penman, M., Rayburn, H., Herz, J., Krieger, M. 1997b. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL)

receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. USA* 94:12610-12615

Ritter, M. C., Scanu, A. M. 1977. Role of apolipoprotein A-I in the structure of human serum high density lipoproteins: reconstitution studies. *J. Biol. Chem.* 252:1208-1216

Robins, S. J., Fasulo, J. M. 1997. High density lipoproteins, but not other lipoproteins, provide a vehicle for sterol transport to bile. *J. Clin. Invest.* 99:380-384

Rogler, G., Trumbach, B., Klima, B., Lackner, K. J., Schmitz, G. 1995. HDL-mediated efflux of intracellular cholesterol is impaired in fibroblasts from Tangier disease patients. *Arterioscler. Thromb. Vasc. Biol.* 15:683-690

Roma, P., Gregg, R. E., Meng, M. S., Ronan, R., Zech, L. A., *et al.* 1993. In vivo metabolism of a mutant form of apolipoprotein A-I, apo A-IMilano, associated with familial hypoalphalipoproteinemia. *J. Clin. Invest.* 91:1445-1452

Ross, R. 1986. The Pathogenesis of Atherosclerosis - An Update. *N. Engl. J. Med.* 314:488-500

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-809

Rothblat, G. H., Mahlberg, F. H., Johnson, W. J., Phillips, M. C. 1992. Apolipoproteins, membrane cholesterol domains, and the regulation of cholesterol efflux. *J. Lipid Res.* 33:1091-1097

Rubin, E. M., Krauss, R. M., Spangler, E. A., Verstuyft, J. G., Clift, S. M. 1991. Inhibition of early atherogenesis in transgenic mice by human apolipoprotein AI. *Nature* 353:265-267

Rye, K.-A. 1990. Interaction of apolipoprotein A-II with recombinant HDL containing egg phosphatidylcholine, unesterified cholesterol and apolipoprotein A-I. *Biochim. Biophys. Acta* 1042:227-236

Savenkova, M. I., Mueller, D. M., Heinecke, J. W. 1994. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *J. Biol. Chem.* 269:20394-20400

Schagger, H., Jagow, G. v. 1987. Tricine-Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379

Schell, W. D., Myers, J. N. 1997. Regression of atherosclerosis: a review. *Prog. Cardiovasc. Dis.* 39:483-496

Schonfeld, G., Pfleger, B., Roy, R. 1975. Structure of human high density lipoprotein reassembled in vitro. *J. Biol. Chem.* 250:7943-7950

Schultz, J. R., Verstuyft, J. G., Gong, E. L., Nichols, A. V., Rubin, E. M. 1993. Protein composition determines the anti-atherogenic properties of HDL in transgenic mice. *Nature* 365:762-764

Seccia, M., Albano, E., Maggi, E., Bellomo, G. 1997. Circulating autoantibodies recognizing peroxidase-oxidized low density lipoprotein. *Arterioscler. Thromb. Vasc. Biol.* 17:134-140

Sevanian, A., Hodis, H. N., Hwang, J., McLeod, L. L., Peterson, H. 1995. Characterization of endothelial cell injury by cholesterol oxidation products found in oxidized LDL. *J. Lipid Res.* 36:1971-1986

Small, D. M. 1988. Progression and regression of atherosclerotic lesions: insights from lipid physical biochemistry. *Arterioscler.* 8:103-129

Stampfer, M. J., Sacks, F. M., Salvini, S., Willett, W. C., Hennekens, C. H. 1991. A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. *N. Engl. J. Med.* 325:373-381

Stein, Y., Stein, O., Dabach, Y., Hollander, G., Ben-Naim, M., Halperin, G. 1996. Anti-atherogenicity of high density lipoprotein. *Isr. J. Med. Sci.* 32:503-508

Steinbrecher, U. P. 1997. Dietary antioxidants and cardioprotection - fact or fallacy? *Can. J. Physiol. Pharmacol.* 75:228-233

Tabas, I., Tall, A. R. 1984. Mechanism of the association of HDL3 with endothelial cells, smooth muscle cells, and fibroblasts. *J. Biol. Chem.* 259:13897-13905

Tajima, S., Yokoyama, S., Yamamoto, A. 1983. Effect of lipid particle size on association of apolipoproteins with lipid. *J. Biol. Chem.* 258:10073-10082

Tall, A. R. 1993. Plasma cholesteryl ester transfer protein. *J. Lipid Res.* 34:1255-1274

Tangirala, R. K., Casanada, F., Miller, E., Witztum, J. L., Steinberg, D., Palinski, W. 1995. Effect of the antioxidant N,N'-diphenyl-1,4-phenylenediamine (DPPD) on atherosclerosis in apoE-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* 15:1625-1630

Theret, N., Delbart, C., Aguie, G., Fruchart, J. C., Vassaux, G., Ailhaud, G. 1990. Cholesterol efflux from adipose cells is coupled to diacylglycerol production and protein kinase C activation. *Biochem. Biophys. Res. Commun.* 173:1361-1368

Vadiveloo, P. K., Fidge, N. H. 1990. Studies on the interaction between apolipoprotein A-II-enriched HDL3 and cultured bovine aortic endothelial (BAE) cells. *Biochim. Biophys. Acta* 1045:135-141

van Tornout, P., Caster, H., Lievens, M.-J., Rosseneu, M., Assman, G. 1981. In vitro interaction of human HDL with human apolipoprotein A-II. *Biochim. Biophys. Acta* 663:630-636

Vogel, R. A. 1997. Coronary risk factors, endothelial function, and atherosclerosis: a review. *Clin. Cardiol.* 20:426-432

vonEckardstein, A. 1996. Cholesterol efflux from macrophages and other cells. *Cur. Opinion Lipidol.* 7:308-319

vonEckardstein, A., Castro, G., Wybranska, I., Theret, N., Duchateau, P., *et al.* 1993. Interaction of reconstituted high density lipoprotein discs containing human apolipoprotein A-I (apoA-I) variants with murine adipocytes and macrophages. *J. Biol. Chem.* 268:2616-2622

vonEckardstein, A., Huang, Y., Wu, S., Funke, H., Noseda, G., Assmann, G. 1995. Reverse cholesterol transport in plasma of patients with different forms of familial HDL deficiency. *Arterioscler. Thromb. Vasc. Biol.* 15:691-703

Walter, M., Gerdes, U., Seedorf, U., Assman, G. 1994a. The high density lipoprotein- and apolipoprotein A-I-induced mobilization of cellular cholesterol is impaired in fibroblasts from Tangier disease subjects. *Biochem. Biophys. Res. Commun.* 205:850-856

Walter, M., Kerber, S., Fechtrup, C., Seedorf, U., Breithardt, G., Assmann, G. 1994b. Characterization of atherosclerosis in a patient with familial high-density lipoprotein deficiency. *Atheroscler.* 110:203-208

Wang, W.-Q., Gustafson, A. 1994. Erythrocyte lipid extraction in alcohol-chloroform systems: a comparative study. *Acta Chem. Scand.* 48:753-758

Watson, K. E., Demer, L. L. 1996. The atherosclerosis-calcification link? *Cur. Opinion Lipidol.* 7:101-104

Weisgraber, K. H., Mahley, R. W. 1980. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J. Lipid Res.* 21:316-325

Westman, J., Roobol, C., Heymans, C., Carlson, L. A., Wulfert, E. 1993. Efflux of cholesterol from cholesterol loaded macrophages by incubation with synthetic HDL-particles. *Scand. J. Clin. Lab. Invest.* 53:773-782

Witztum, J. L., Horkko, S. 1997. The role of oxidized LDL in atherogenesis: immunological response and anti-phospholipid antibodies. *Ann. N. Y. Acad. Sci.* 811:88-99

Yla-Herttuala, S., Palinski, W., Butler, S. W., Picard, S., Steinberg, D., Witztum, J. L. 1994. Rabbit and human atherosclerotic lesions contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler. Thromb.* 14:32-40

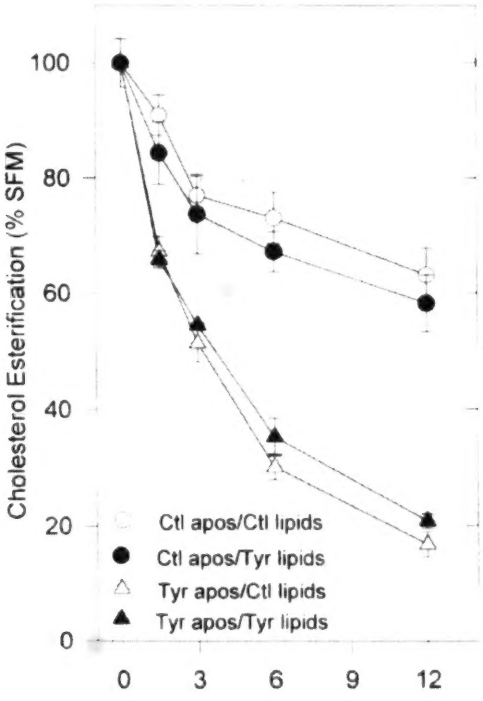
Yla-Herttuala, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., *et al.* 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* 84:1086-1095

Yokota, T., Hansson, G. K. 1995. Immunological mechanisms in atherosclerosis. *J. Intern. Med.* 238:479-489

Zhao, Y., Sparks, D. L., Marcel, Y. L. 1996a. Effect of the apolipoprotein A-I and surface lipid composition of reconstituted discoidal HDL on cholesterol efflux from cultured fibroblasts. *Biochemistry* 35:16510-16518

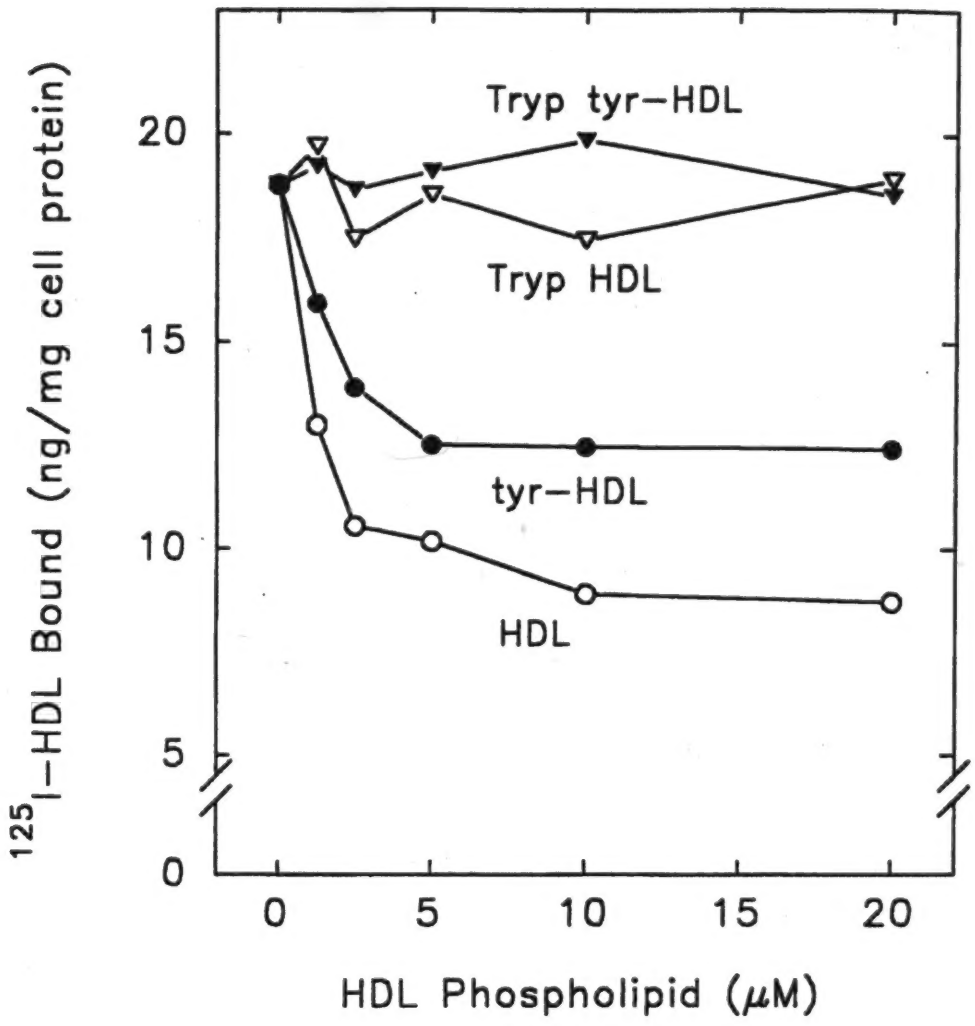
Zhao, Y., Sparks, D. L., Marcel, Y. L. 1996b. Specific phospholipid association with apolipoprotein A-I stimulates cholesterol efflux from human fibroblasts. *J. Biol. Chem.* 271:25145-25151

Appendix 1: Reconstituted HDL prepared using apolipoproteins and whole lipid extracts from HDL and tyrosylated HDL. Preliminary results of experiments by Dr. Wen-Qi Wang.



Apolipoproteins isolated from tyrosylated HDL and reconstituted with whole lipids have a greater ability to deplete cellular cholesterol than apolipoproteins isolated from control HDL. Cholesterol-loaded human fibroblasts were incubated for 16 h with the indicated concentration of reconstituted HDL containing the indicated apolipoprotein and lipid fractions. The cells were then washed and incubated with [^{14}C]oleate for 1 h to measure incorporation into cholesteryl ester. Abbreviations: Ctrl apo, apolipoproteins isolated from HDL; Tyr apo, apolipoproteins isolated from tyrosylated HDL; Ctrl lipid, whole lipid extract from HDL; Tyr lipid, whole lipid extract from tyrosylated HDL. Values are the mean +/- SD of 4 determinations.

Appendix 2: Effects of protease treatment of HDL and tyrosylated HDL on their ability to compete for high-affinity HDL binding sites on the surface cholesterol-loaded fibroblasts. Cholesterol-loaded human fibroblasts were incubated with intact HDL or trypsin-treated (30 minutes) control or tyrosylated HDL for 3h at 4°C, washed, and incubated with [^{125}I]HDL for 1h.

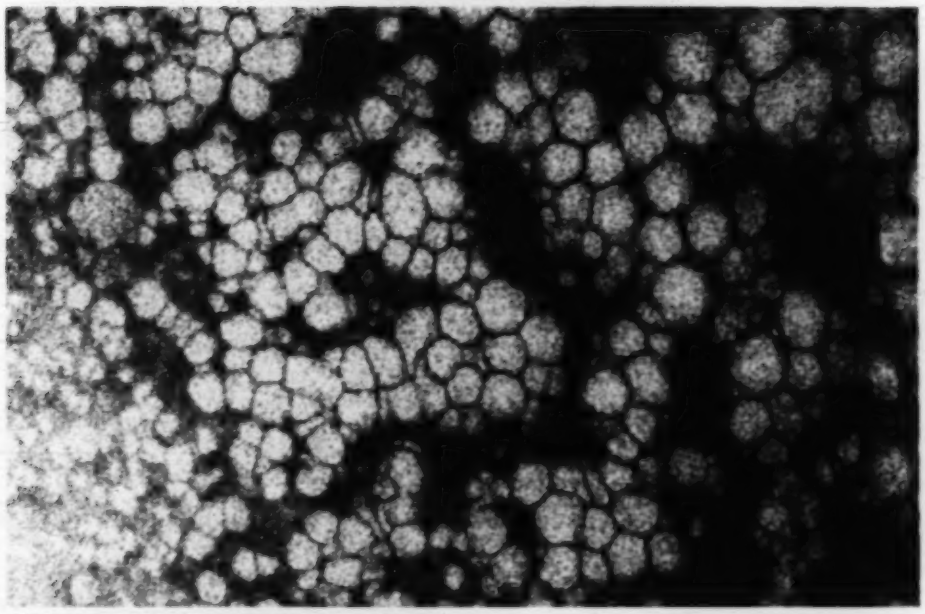

Tryp tyr–HDL
Tryp HDL
tyr–HDL
HDL
20
15
10
5
0
0
5
10
15
20
^{125}I–HDL Bound (ng/mg cell protein)
HDL Phospholipid (μM)

Appendix 3: Transmission electron micrographs taken in collaboration with Dr. Ming Chen (Surgical-Medical Research Institute, University of Alberta, Edmonton, AB).

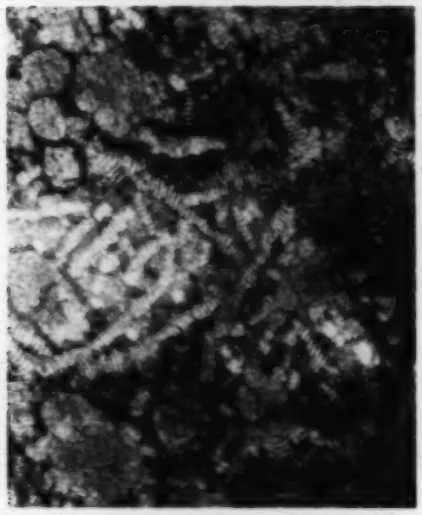
A. Reconstituted HDL prepared using apolipoproteins from control HDL and commercial PC and FC, final molar ratio 80:25:1, PC:FC:apolipoprotein. Magnification: 24,000x. Stained using phosphotungstic acid.

B. Reconstituted HDL prepared using apolipoproteins from tyrosylated HDL and the whole lipid extract from tyrosylated HDL, final molar ratio 80:1, phospholipid: apolipoprotein. Magnification: 250,000x (1" = 100 nm).Stained using phosphotungstic acid.

Appendix 3: A and B



B



A

C. Chemical composition of reconstituted particles - data from Dr. Wen-Qi Wang.

I. Concentration of protein and lipids in r-HDL (mg/mL)

r-HDL constitution	Apolipoprotein	Total Phospholipids	CE	FC	TG
Control protein / control lipids	0.629	0.580	0.031	0.019	0.028
Control protein / tyrosylated lipids	0.759	0.605	0.013	0.027	0.015
Tyrosylated protein / control lipids	0.547	0.481	0.008	0.032	0.015
Tyrosylated protein / tyrosylated lipids	0.585	0.537	0.018	0.027	0.023

II. Concentration of protein and lipids in r-HDL (mM)

r-HDL constitution	Apolipoprotein	Total Phospholipids	CE	FC	TG
Control protein / control lipids	22.50	737.80	47.60	49.50	31.60
Control protein / tyrosylated lipids	27.10	769.60	20.00	70.70	16.60
Tyrosylated protein / control lipids	19.50	611.90	12.30	81.90	17.40
Tyrosylated protein / tyrosylated lipids	20.90	683.10	27.60	69.10	26.10

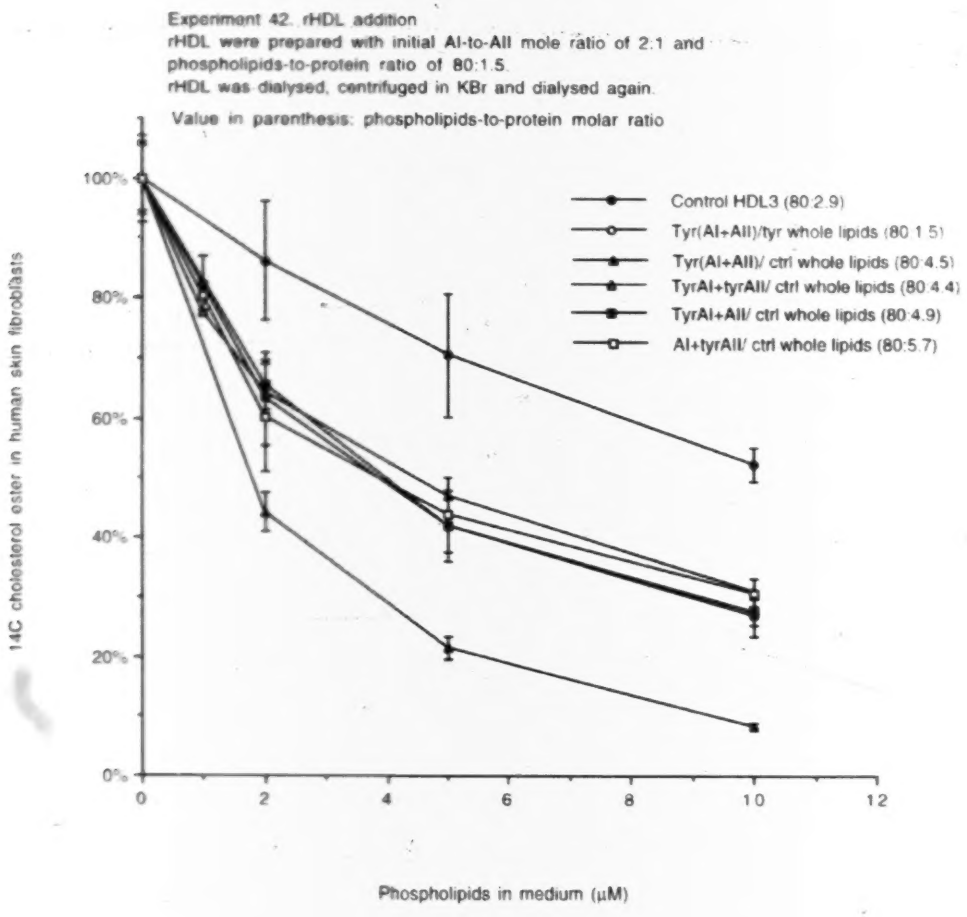
III. Molar ratios of protein to lipids in r-HDL

r-HDL constitution	Apolipoprotein	Total Phospholipids	CE	FC	TG
Control protein / control lipids	1	32.90	2.10	2.20	1.40
Control protein / tyrosylated lipids	1	28.40	0.70	2.60	0.60
Tyrosylated protein / control lipids	1	31.30	0.60	4.20	0.90
Tyrosylated protein / tyrosylated lipids	1	32.70	1.30	3.30	1.20

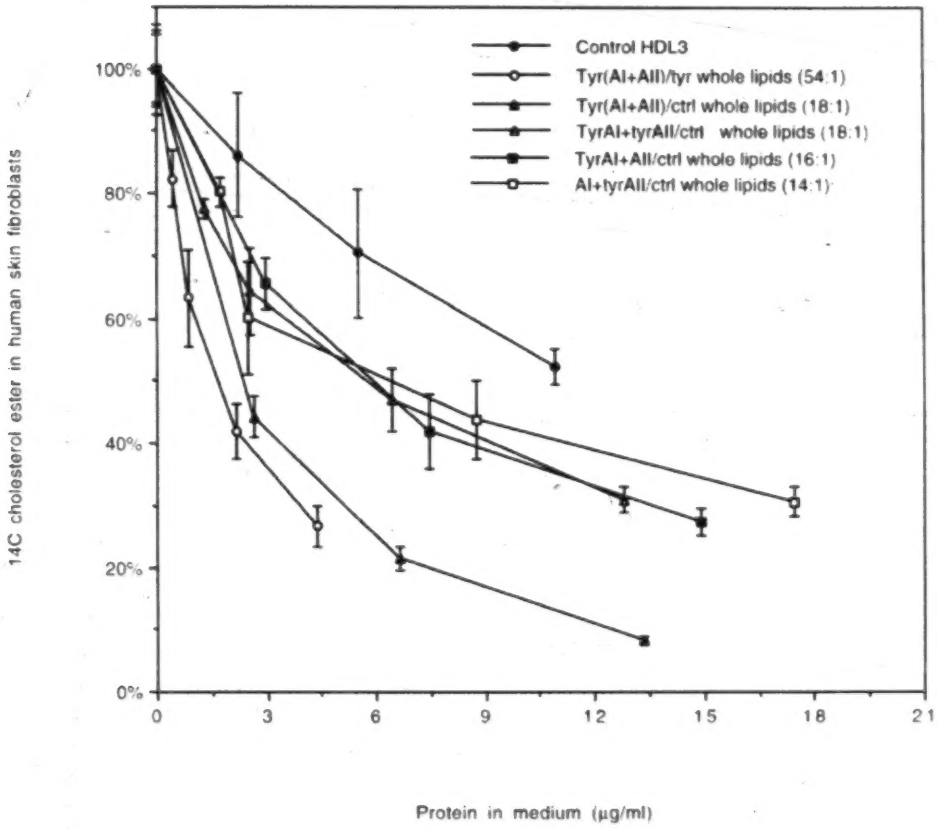
IV. Recovery of protein or lipids (% of initial added amounts)

r-HDL constitution	Apolipoprotein	Total Phospholipids	CE	FC	TG
Control protein / control lipids	71.60%	60.00%	54.80%	68.20%	60.30%
Control protein / tyrosylated lipids	93.20%	67.60%	49.20%	77.10%	60.20%
Tyrosylated protein / control lipids	76.20%	60.90%	17.10%	135.50%	41.30%
Tyrosylated protein / tyrosylated lipids	81.80%	68.40%	78.90%	85.90%	108.00%

Appendix 4: Reconstituted HDL prepared using mixtures of unoxidized and tyrosylated apo A-I and apo A-II, and whole lipid extract from HDL. Preliminary results of experiments by Dr. Wen-Qi Wang.



Experiment 42. rHDL addition
rHDL were prepared with initial AI-to-AII mole ratio of 2:1 and phospholipids-to-protein ratio of 80:1.5.
rHDL was dialysed, centrifuged in KBr and dialysed again.
Value in parenthesis: phospholipids-to-protein molar ratio



Appendix 5: Western blot analysis of control and tyrosylated HDL

Methodology

Immunoblotting consists of two steps: transfer of proteins from the gel to a nitrocellulose membrane by electrophoresis, and detection of proteins of interest using specific antibodies.

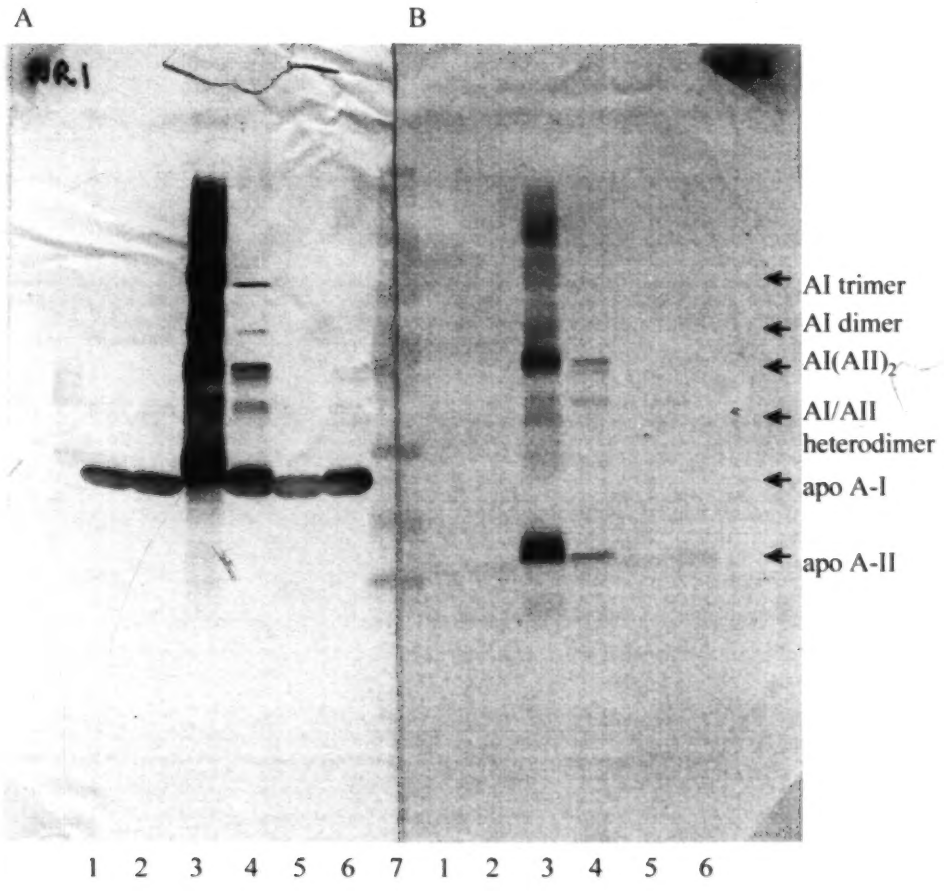
Transfer conditions

Proteins were transferred from the SDS acrylamide gel to a nitrocellulose membrane using a Bio-Rad Trans-Blot Cell containing transfer buffer (25 mM Tris, 0.2 M glycine, 20% (v/v) ethanol) at ~4 °C. Transfer buffer containing ethanol was chosen because ethanol prevents swelling of the gel and facilitates protein binding to nitrocellulose. A constant current of 420 mA was maintained for 4 hours. Success of transfer was checked by staining with Ponceau S, a nonpermanent, removable protein dye.

Blocking, washes, incubation with antibodies, and detection

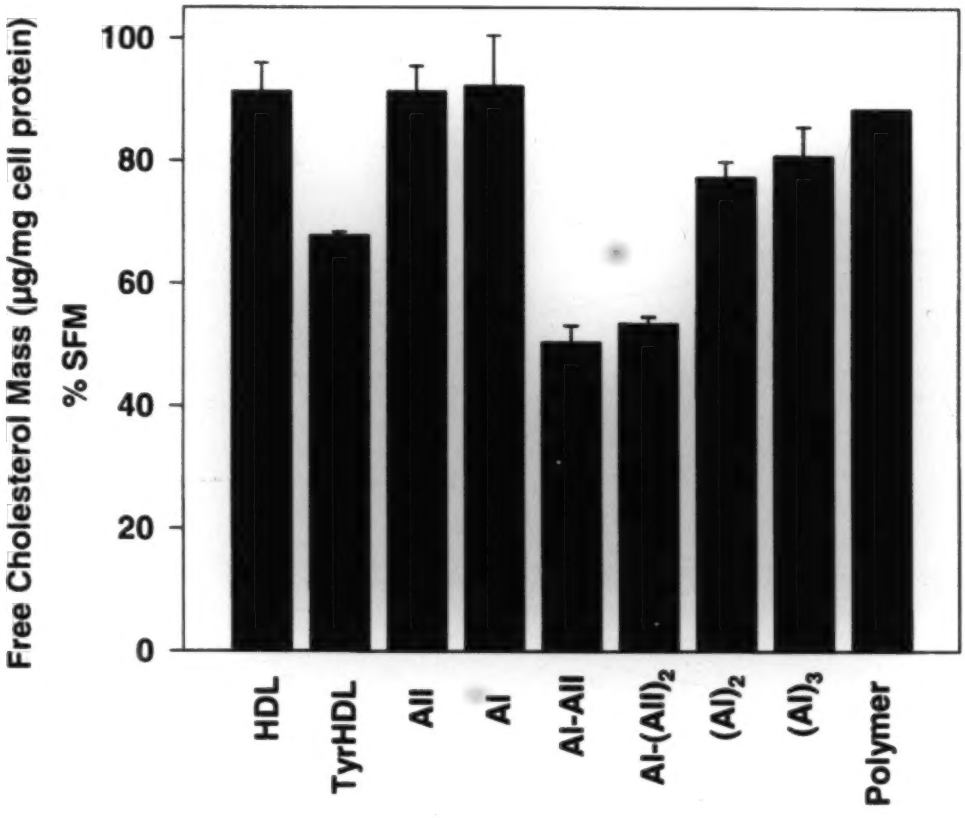
Once the proteins have been transferred from the polyacrylamide gel to the nitrocellulose membrane, the membrane was incubated with blocking buffer (25 mM Tris, 140 mM NaCl, pH 7.5 containing 1% (w/v) skim milk powder) for 1 hour . The blocking step ensured that unoccupied regions of the nitrocellulose membrane are bound by nonreactive proteins, preventing nonspecific binding of antibodies to the membrane. The membrane

was washed with 25 mM Tris, 140 mM NaCl, 0.05% (w/v) Tween 20, pH 7.5, then incubated overnight in blocking buffer with a primary antibody (diluted 1:1000), which specifically recognizes the protein of interest. The membrane was washed with 25 mM Tris, 140 mM NaCl, 0.05% (w/v) Tween 20, pH 7.5, then incubated for 2 hours in blocking buffer with a secondary antibody (diluted 1:5000), which recognizes the constant region of the primary antibody. The secondary antibody is conjugated to horseradish peroxidase, which may be reacted with a substrate to give either a visible product or one which can be detected using X-ray film.



Appendix 5. Western blot analysis of tyrosylated HDL. A, anti-apo A-I primary antibody; B, anti-apo A-II primary antibody. Lanes: 1, HDL incubated at 4°C for 24 h; 2, HDL incubated at 37°C for 24 h; 3, HDL modified by incubation at 37°C for 24 h with complete peroxidase/H_20_2/L-tyrosine system; 4, complete system without L-tyrosine; 5, complete system without peroxidase; 6, complete system plus catalase (5 nM); 7, molecular weight markers. Identities of apolipoproteins are indicated.

Appendix 6: Reconstituted HDL prepared using crosslinked apolipoprotein species purified from Tyr-HDL by preparative electrophoresis and whole gel elution. Results of experiments by Audric Moses and Dr. Wen-Qi Wang.



Depletion of cellular free cholesterol mass by r-HDL containing tyrosylated HDL apo species. Cholesterol-depleted human fibroblasts were incubated with 50 µg/mL LDL and 20 µg/mL r-HDL containing the indicated tyrosylated HDL apo species. Cellular lipids were extracted and free cholesterol determined. Values are the mean +/- SD of 4 determinations. Free cholesterol mass in SFM cells was 21.5 +/- 1.8 µg/mg cell protein.